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POLYDACTYLY, RELATED DEFECTS AND AXIAL SHIFTS—A CRITIQUE

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On occasion an hypothesis of such appeal is advanced in explanation of a particular phenomenon that one is reluctant to tamper with it. Yet, when the evidence indicates that the hypothesis is erroneous, one must subject it to criticism in order to restore proper perspective. Ideally such criticism should be followed by a properly supported alternate explanation of the phenomenon. An ingenious hypothesis was formulated by T. C. Carter in 1954 in order to account for a number of anomalies which result from the action of the luxate mutation of the house mouse. In general homozygotes are characterized by tibial hemimelia (distal end of tibia either missing or defective) which may or may not be accompanied by preaxial polydactyly or, more rarely, by ectrodactyly (absence of digits). Heterozygotes are characterized by preaxial polydactyly of the hind limbs. Kidney defects and reduction in the number of lumbar vertebrae may be found in both homo- and heterozygotes; most frequently in the former and usually only in heterozygotes with the more extreme expression of the luxate mutation. Background genotype exerts a considerable influence on the expression of the syndrome.

The fact that the more severe expression of the luxate syndrome was accompanied by a reduction in the number of lumbar vertebrae (i.e., the hind limbs were farther anterior than normal) was so striking that Carter made this observation the basis of his hypothesis as follows: Limb development results from the interaction of two tissues, one which induces the limb (ectoderm) and the other which responds to the limb-forming stimulus (mesoderm). The limb-potent tissue is restricted to a region in the neighborhood of a few specified somites. The inductor extends along most, but not all, of the limb-potent tissue. The luxate mutation produces its effects by shifting the limb inductor in an anterior direction. The shift is slight in heterozygotes and more pronounced in homozygotes. The slight craniad displacement of the inductor puts it in contact with a region of the limb-potent tissue which ordinarily is not involved in limb formation and which produces a "poorly controlled" limb-forming response and consequent preaxial poly-

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dactyly. With greater craniad displacement of the limb inductor, as in homozygotes, its anterior end is in contact with tissue which is devoid of limb potency. This accounts for the tibial hemimelia.

The hypothesis requires that the limb inductor and the limb-potent tissue be fairly precisely aligned with respect to each other. In a private communication in which Carter further elaborated his hypothesis he states: "The position of the limb relative to the body axis is determined primarily by the position of the inductor (E). If along any part of E there is no juxtaposed limb-potent tissue (M), the corresponding part of the limb tends to be absent; but if the misalignment of E and M is only slight, there may be (a) no apparent effect, or (b) a slight narrowing of the limb proximally followed by a compensating excess distally, manifesting itself as polydactyly." The experiments of Saunders (1948) suggested to Carter that the limb inductor and limb-potent tissue "may be the precursors of, respectively, the apical ectodermal ridge and the lateral mesenchyme at hindlimb level." [Unfortunately, I misquoted Carter (Zwilling, 1956c) and stated that he believed the apical ridge itself to be the limb inductor. As noted above he, in reality, speaks of the *precursor* of the ridge as the possible inductor.] After a full elaboration of this hypothesis two questions are asked: "Are ectrodactyly and hemimelia in other material associated with a displacement of the limb buds along the body axis? Does the lateral mesoderm immediately adjacent to a limb bud lack limb potency?" The presumption is that affirmative answers to these queries would support the hypothesis.

Both of the questions which Carter posed may now be answered in the affirmative. Forsthoefel (1953, 1957) and Green (1955) have described a luxoid mutation in mice whose features are essentially the same as those of the luxate mice except that the fore limbs are also involved and there is an increased number of presacral vertebrae. These authors have adopted Carter's hypothesis and explain the luxoid syndrome as due to a posterior displacement of the limb-potent tissue (mesoderm). Thus the luxate syndrome is thought to be the result of a craniad shift of the inductor (ectoderm) and the luxoid syndrome the results of a caudad shift of the limb-potent tissue (mesoderm).

The mesoderm immediately adjacent to chick embryo limb buds has been tested for limb potency in this laboratory (unpublished). This was done by placing such mesoderm into the ectodermal jackets from a limb bud where it was in contact with the apical ridge. These "limb buds" were grafted to host embryos (see Zwilling, 1955, for procedure). There was no limb outgrowth; this failure could be traced to the inability of the non-limb mesoderm to support the apical ridge of ectoderm.

Recently we have found that there is a decrease in the number of presacral vertebrae in a diplopod (*dp-2*) mutation discovered (Landauer, 1956a) in Black Minorca chickens (table 1). On the other hand our data reveal that there was no detectable shift in the position of the sacrum in the *py* mutation of pigeons (Hollander and Levi, 1942) (material kindly provided

TABLE 1

DIPLOPODS

No. of presacral vertebrae	Polydactyls	Controls
26	10	1
27	0	15
Total	10	16
	Mean = 26 = 0	Mean = 26.94 = .06
	Standard error of difference = .06	

by Dr. W. F. Hollander, Department of Genetics, Iowa State College, Ames, Iowa) or the duplicate (*Pod*) mutation of chickens originally described by Warren (1941).

DISCUSSION AND CRITIQUE

Despite the fact that both of Carter's "crucial" questions can be answered in the affirmative we feel that his hypothesis is not tenable as an explanation of the effects of the luxate and luxoid mutations. Enough information has accumulated since 1954 so that his ideas may be re-evaluated with respect to what is known about limb development. Let us emphasize that all of our information comes from experiments performed upon chick or amphibian embryos. There has been no direct experimentation with the mouse, and in this discussion one must accept the supposition that essentially the same developmental phenomena seen in the lower vertebrates exist in mammals. That no major discrepancies exist between amphibia and birds in regard to limb development has been emphasized by the recent experiments of Tschumi (1956, 1957) on limb development in Xenopus.

Shift hypothesis and limb buds

Let us first see whether Carter's hypothesis can be applied to the limb bud itself. According to the hypothesis the apical ectodermal ridge would be responsible for limb induction and the limb-bud mesoderm would be the reacting "limb-potent" tissue. The limb pattern (as indicated by the number of digits) should be dependent on the inductive activity of the ridge. A slight craniad displacement of the ridge of the hind limb bud relative to its mesoderm should result in the induction of supernumerary digits from the normally inactive limb-potent tissue anterior to the limb inductor. Carter accounted for the order in which parts of the limb disappear by an extrapolation of Saunders' (1948) fate map of the chick embryo wing bud to the mouse hind limb bud. The long axis of the presumptive long bones is oblique in the limb bud: the presumptive humerus points postaxially, the presumptive radius and ulna point preaxially, etc. (see diagram on page 33 of Carter's paper). The supposition is that if the disposition of presumptive elements in the mouse hind limb were the same then a craniad displacement

of the ectodermal ridge to a region where its anterior end is in contact with non-limb-potent mesoderm would result in the absence of the proximal end of the humerus (femur), distal end of the radius (tibia), and so forth.

Carter was correct in assuming an interaction between the ectodermal ridge and the limb bud mesoderm. However, he was incorrect in assuming that the ectodermal ridge plays a dominant role in the induction. The ectodermal ridge is indeed responsible for outgrowth induction (Saunders, 1948; Saunders, Gasseling and Cairns, 1955, 1957; Zwilling, 1955). However, the ridge is quite dependent, under most circumstances, on the mesoderm. The very existence of the ridge as an active inducer of limb outgrowth is dependent on the presence of a maintenance factor in the mesoderm. More of the ridge than is normally active in inducing outgrowth is capable of doing so. This is shown by a number of experiments in which the ridge was displaced but in which there was normal limb development nevertheless. The activity of the ridge conformed to the pattern of distribution of the maintenance factor in the mesoderm (Zwilling, 1956a, b). Experiments with polydactylous limb buds illustrate this point even more clearly. When mesoderm from genetically polydactylous limb buds was covered by ectoderm from genetically normal buds the region of active ectodermal ridge became more extensive than in a normal limb. Just as in typical polydactylous limb buds, this resulted in a greater preaxial outgrowth and consequent excess digital development (Zwilling and Hansborough, 1956). The pattern of the limb, therefore, resulted from a difference in the distribution of the maintenance factor which led to a response in the ectodermal ridge, which in turn resulted in a more extensive preaxial outgrowth.

Carter's argument about the oblique position of the presumptive long bone elements has recently been vitiated too. Hampé (1956) has mapped the presumptive areas of the hind limb bud of the chick embryo with the same procedures used by Saunders. The presumptive femoral, tibial, etc. areas do indeed have an oblique disposition, but opposite to that found in the homologous wing structure! Thus the distal end of the presumptive tibia points postaxially and not in a preaxial direction. According to Carter's reasoning this would, therefore, lead one to expect defects of the proximal end of the tibia instead of the distal defects actually encountered.

Let us now see what we may expect from a craniad displacement of the ectodermal ridge relative to the limb bud mesoderm. A slight displacement should have no effect, since the ridge would merely adjust to the maintenance factor in the mesoderm. A more extensive displacement should leave the *postaxial* end of the mesoderm without an outgrowth inducer—and should produce *postaxial* deficiencies. A marked caudal shift of the ectoderm (or craniad displacement of the mesoderm) might result in preaxial deficiencies; but these would be apparent first as distal defects of the femur and then, in more extreme cases, as proximal defects of the tibia (see remarks about Hampé, 1956, above). This, of course, is not quite what is seen in either the luxate or the luxoid limbs. These arguments, we believe, eliminate Carter's hypothesis as a possible causal mechanism for the luxate effects when it is applied to the limb bud stages.

Shift hypothesis and pre-limb bud stages

There are two requirements of the hypothesis which render it inapplicable to pre-limb bud stages. The first is that both the limb-inducing and the limb-potent components, that is, ectoderm and mesoderm (or, as Carter has pointed out in his letter, any two component tissues) must be of limited extent along the body axis. The second is that the two components must be in fairly precise juxtaposition in order that a limb develop normally (see quote in introduction). These requirements imply that the presumptive limb tissues (limb field) represent a mosaic, that is, a rather strict delimitation of presumptive bone areas both in the inductor and in the limb-potent tissue—hence the emphasis on precise alignment.

The limb field of amphibians has been subjected to extensive investigation. Some of the observed facts, assuming that they may be transferred to the mouse limb field, are incompatible with Carter's requirements. The earliest work of R. G. Harrison and his students revealed that the limb field (i.e., the area capable of forming a limb) is much more extensive than the area which eventually gives rise to the limb. In addition, the ectoderm from most of the early embryo's body is capable of participating in limb formation. Balinsky's (1933) failure, quoted by Carter, to induce supernumerary limbs close to the hind-limb buds may not be a contradiction. Balinsky used artificial inductors and he has recently (1956) ascribed their effect to the elimination of the basement membrane over the area of the induced limb. This may be quite a different phenomenon from the one involved in normal limb induction. In general the experiments with pre-limb bud stages of amphibian embryos indicate that both the limb-potent ectoderm and mesoderm are not restricted to a sharply circumscribed area. We must assume that a similar situation exists in amniotes until contradictory evidence is produced.

Mosaicism in a field

The requirement that the two presumptive limb tissues be precisely aligned for normal limb development, with particular limb defects resulting from a particular misalignment, implies a mosaic structure of the limb field. Such an assumption is not compatible with the properties of a field (Weiss, 1939) which by definition cannot be a mosaic. While much about limb development is still unknown we do know a number of important facts. When a limb field or limb bud is induced, it is not a telescoped miniature which contains presumptive limb structures arranged in a precise way. The chief characteristic of the early developing limb system is that of distal elongation. Elaboration of a limb depends upon the continuation of this elongation. Mapping experiments of Saunders (1948) and Hampé (1956) have revealed that the more distal limb elements are not represented at all by presumptive areas in the earlier limb buds. They appear gradually as the limb elongates. It is obvious that the stem cells which normally give rise to the distal structures must be present in the earliest limb primordium. But their unfolding as definite areas is dependent on continued elongation and de-

velopment of the limb. This is demonstrated by marking experiments as well as those which reveal remarkable regulatory powers of the limb bud tissues. A presumptive proximal area may form distal structures if, in some way, it can be made to lie in a distal region (Saunders, Gasseling and Cairns, 1955, 1957; Zwilling, 1956b), and so on.

Let us, for the sake of argument, assume that the limb field is much more restricted in a mouse embryo than in an amphibian embryo and that the two tissue layers must be in precise juxtaposition to yield a limb bud of normal size and structure. Even under these conditions we must still recognize the non-mosaic nature of the limb field and the important consequences of limb elongation. Bearing these properties in mind we should expect that any but a very extreme displacement of one of the presumptive limb tissues with respect to the other should result in a smaller than normal limb bud. However, the limb pattern should be maintained (see Weiss, 1939) and a normal, albeit small, limb should develop. In light of our current knowledge of limb development this is the most that we can expect from Carter's postulated tissue displacement.

Unitary nature of luxate syndrome

Carter's hypothesis has the merit of postulating a single event for an explanation of most of the observed effects of the luxate mutants. But it is not compatible with what is now known about limb development. The observations of Carter and those of Forsthoefel and Green, as well as our own data for the diplopod mutation, can be taken at face value to indicate that some early event, mediated via a genetic factor, can result in displacement of a limb field. However, the displacement per se need not be the cause of subsequent limb anomalies. Hence we need not expect anomalous limbs each time the limb is displaced nor do we require limb displacement in order to produce limb anomalies.

ALTERNATE HYPOTHESIS

We should like to present evidence that polydactyly and related defects are the consequences of developmental events which occur relatively late and produce their effects via alteration in the distribution of the ectodermal ridge maintenance factor (MF).

According to this view two factors are critical for determining the structural details eventually seen in a limb. The amount of digital excess is determined by the *extent* of the duplication or spread of the MF, while the involvement of proximal parts depends on the *time* when the additional outgrowth is induced. Such a viewpoint is quite consistent with known facts.

Altered limb paddle

The first definite visible effect which precedes an altered digital pattern is the formation of a limb paddle of abnormal size. Accounts of enlarged paddles in early stages of polydactyly have been reviewed by Zwilling and Hansborough (1956); and Tschumi (1954) has reported that the number of

digits which form in Xenopus limbs is proportional to the limb paddle area. His data from experimentally reduced limbs are quite precise. Any hypothesis must, therefore, relate earlier events to the visibly enlarged limb paddle.

It is evident that limb paddle alterations may result from causes which become operative: (a) very early in development, by action on the limb field; (b) somewhat later, when the limb bud is just beginning to take form; or (c) relatively late, after the limb bud is fully formed. Let us examine the possible consequences of events whose actions start at these different times.

a) Action on the limb field

If limb anomalies were the result of very early divergence from normal events we should expect the initial limb buds to be atypical. They should be very large in cases of field duplication or smaller than normal in cases of field contraction. In limb buds of the only polydactylous mutation examined critically for initial size ("Duplicate") there is no dimensional difference from normal buds (Zwilling and Hansborough, 1956). Carter thought that some homozygous luxate limb buds were smaller than normal, but the evidence was not critical. One should also expect, in cases of limb field duplication or enlargement that proximal limb structures and possibly girdle elements should be duplicated or enlarged. This result was actually obtained by Wolff and Kahn (1957 a and b) when they transected the hind limb field in 20–25 somite (approximately 50 hours incubation) chick embryos. In a number of their cases the resulting polydactyly was accompanied by doubling of the tibia and femur. More cases of genetic polydactyly should be examined in early stages for additional evidence; but the data from the duplicate stock, taken with the rareness or total absence of duplication of proximal limb structure, lead us to believe that duplication of a *limb field* is not involved in the development of polydactyly.

b) Action at later stages

Duplication or enlargement of a possible limb-forming center within the buds may be expected to have the following consequences: there should be some duplication of distal structures whether the deviation from normal occurs early or late in the development of the limb bud. In the former situation one should expect some of the proximal structures to be duplicated in addition to the digits. Exactly how far proximad the duplications would extend should depend on *when* the enlarged outgrowth begins, and this, of course, should be related to *when* the mediating factors exert their effects.

Experimental Evidence

Digital pattern may be modified experimentally relatively late in development. Sturkie (1943) found that lowered temperatures most effectively suppressed polydactyly in chickens when applied during the third day of development (after limb buds are well formed). Insulin was most effective

when injected between 72 and 96 hours of incubation (Landauer, 1948). Colchicine treatment of three-day chick embryo limb buds either suppressed or enhanced the expression of the duplicate mutation (Gabriel, 1946). Finally, digital anomalies are found frequently when genetically normal three-day limb buds are grown as grafts in an atypical site. Hypodactyly is encountered most frequently, but on rare occasions polydactyly may occur (Hamburger, 1938, and our own experiments).

Recently Saunders (in press, J. Exp. Zool.) obtained a high incidence of distal duplications of wings.* These results followed operations in which the distal halves of genetically normal three-day wing buds were rotated and replaced on the proximal halves in such a way that the antero-posterior axis was inverted 180°. There was no duplication following inversion of entire wing buds. Our own experiments (Zwilling, 1956c) led us to postulate that an enlarged limb paddle was the consequence of an atypical distribution of the maintenance factor discussed above. Saunders accepts this and interprets his results to mean that the original region of high MF activity persists in the inverted part of the wing bud and induces one major outgrowth center, while the high MF activity of the original center of the base is transmitted to the overlying graft and induces a second outgrowth center.

All the evidence cited above is consistent with the idea that polydactyly results from interactions which affect the developing limb system relatively late, after the limb bud as such has formed.

Tibial hemimelia and radius duplication

Polydactyly may be associated with defects of the long bones immediately proximal to the digits. The tibial hemimelia characteristic of luxate homozygotes has been mentioned above. In duplicate chickens polydactyly may be accompanied by either deficiencies or duplication of the radius, or both (Landauer, 1956b). Thus there may be two *distal* radial fragments both of which are deficient proximally! The increased amount of tissue involved in the excess limb outgrowth probably comes both from increased cell proliferation and from an actual movement of cells from proximal regions into the region of active outgrowth. The seeming paradox of a digital excess accompanied by tibial deficiency may result from an excess outgrowth in luxate limbs which occurs relatively late, involves only the digital area, and attracts some of the tissue immediately proximal to the area of excess outgrowth. The key to whether proximal deficiencies will be encountered is *the time* of onset of excess outgrowth. If it starts early there will be a sufficient base for outgrowth and relatively little competition for

*Note added to proof: Since this was written Saunders' paper has been published: Saunders, J. W., Jr., M. T. Gasseling and Sr. M. D. Gfeller, 1958, Interactions of ectoderm and mesoderm in the origin of axial relationships in the wing of the fowl, J. Exp. Zool. 137: 39–74; and independent confirmatory evidence has appeared: Ambrino, R. and M. Camaso, 1958, Experimental observations on influences exerted by the proximal over the distal territories of the extremities. Experientia 14: 241–243.

proximal tissues; if excess outgrowth starts later, proximal tissues may be attracted to the region of outgrowth.

Ectromelia and amelia

According to our interpretation, ectromelia may be due to the contraction of the MF gradient. Amelia (as in wingless chicks) would be due to the absence of an MF center.

Causal factors

Unfortunately, we have no clues about the nature of the biochemical and physical events whose disturbance leads to polydactyly and related effects. We are reasonably certain that relative displacement of tissue layers is not one of the features of such disturbances. It is more likely, in view of the list of treatments which modify the expression of polydactyly, that alteration of one or another of the important metabolic sequences is the key to the production of these limb defects. Attempts to find a "unitary action" for gene expression should in many cases, be directed at the metabolic events instead of relying solely on morphological relationships. About all that can be said of the luxate syndrome from this point of view is that factors which alter the position of a limb field may, if they last long enough and are of the right sort, produce any of the limb conditions discussed above.

SUMMARY

Data are presented which show that the hind limbs of diplopod chick embryos (a polydactylous mutation) are one vertebra further anterior than normal. No displacement of the hind limb was encountered in a polydactylous pigeon mutation or in the duplicate mutation of chickens. Carter's hypothesis for the development of the luxate syndrome of mice is discussed and criticized. It is shown to be incompatible with known facts about limb development; and an alternate hypothesis is advanced, which postulates that polydactyly and related defects result from relatively late deviations in the distribution of an hypothetical ectodermal ridge maintenance factor believed to be present in limb bud mesoderm.

LITERATURE CITED

Balinsky, B. I., 1956, A new theory of limb induction. Proc. Nat. Ac. Sci. 42: 781–785.

Carter, T. C., 1954, Genetics of luxate mice. IV. Embryology. J. Genet. 52: 1–35.

Forsthoefel, P. F., 1953, Developmental genetics of luxoid, a new skeletal variation in the house mouse, Mus musculus. Genetics 38: 661–662 (Abstr.).

1957, Further studies on the effects of the luxoid gene on the skeleton of the mouse, including interaction with the luxate gene. Genetics 42: 370 (Abstr.).

Gabriel, M., 1946, The effect of local application of colchicine on Leghorn and polydactylous chick embryos. J. Exp. Zool. 101: 339–350.

Green, M. C., 1955, Luxoid—a new hereditary leg and foot abnormality in the house mouse. J. Hered. 46: 91–99.

Hamburger, V., 1938, Morphogenetic and axial self-determination of transplanted limb primordia of 2-day chick embryos. J. Exp. Zool. 77: 379–399.

Hampé, A., 1956, Sur la topographie des ébauches présomptives du membre postérieur du Poulet. C. R. Acad. Sci. 243: 970–973.

Hollander, W. F. and W. M. Levi, 1942, Polydactyly, a sublethal character in the pigeon. J. Hered. 33: 385–391.

Landauer, W., 1948, The phenotypic modification of hereditary polydactylism of fowl by selection and by insulin. Genetics 33: 133–157.

1956a, A second diplopod mutation of the fowl. J. Hered. 47: 57–63.

1956b, Rudimentation and duplication of the radius in the duplicate mutant form of fowl. J. Genet. 54: 199–218.

Saunders, J. W., Jr., 1948, The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. J. Exp. Zool. 108: 363–404.

Saunders, J. W., Jr., M. T. Gasseling and J. M. Cairns, 1955, Effect of implantation site on the development of an implant in the chick embryo. Nature 175: 673–675.

Saunders, J. W., Jr., J. M. Cairns and M. T. Gasseling, 1957, The role of the apical ridge of ectoderm in the differentiation of the morphological structure and inductive specificity of limb parts in the chick. J. Morph. 101: 57–87.

Sturkie, P. D., 1943, Suppression of polydactyly in the domestic fowl by low temperature. J. Exp. Zool. 93: 325–346.

Tschumi, P. A., 1954, Konkurrenzbendingte Rückbildungen der Hinterextremität von Xenopus nach Behandlung mit einem Chloraethylamin. Rev. Suisse de Zool. 61: 177–270.

1956, Die Bedeutung der Epidermisleiste für die Entwicklung der Beine von Xenopus laevis Daud. Rev. Suisse de Zool. 63: 707–716.

1957, The growth of the hind limb bud of Xenopis laevis and its dependence upon the epidermis. J. Anat. 91: 149–173.

Warren, D. C., 1941, A new type of polydactyly in the fowl. J. Hered. 32: 3–5.

Weiss, P., 1939, Principles of Development. Henry Holt and Co., New York.

Wolff, E. and J. Kahn, 1947, Production expérimentale de la polydactylie chez l'embryon d'oiseau. C. R. Acad. Sci. 224: 1583–1584.

1947, La régulation de l'ébauche des membres chez les oiseaux. C. R. Soc. Biol. 141: 915–916.

Zwilling, E., 1955, Ectoderm-mesoderm relationship in the development of the chick embryo limb bud. J. Exp. Zool. 128: 423–442.

1956a, Interaction between ectoderm and mesoderm in the chick embryo. I. Axis establishment. J. Exp. Zool. 132: 157–172.

1956b, Ibid. II. Experimental limb duplication. J. Exp. Zool. 132: 173–187.

1956c, Reciprocal dependence of ectoderm and mesoderm during chick embryo limb development. Am. Nat. 90: 257–265.

Zwilling, E. and L. A. Hansborough, 1956, Interaction between limb bud ectoderm and mesoderm in the chick embryo. III. Experiments with polydactylous limbs. J. Exp. Zool. 132: 219–239.

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INHERITANCE IN NICOTIANA TABACUM XXIX: THE RELATIONSHIP OF RESIDUAL CHROMOSOME HOMOLOGY TO INTERSPECIFIC GENE TRANSFER

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Reduction in chromosomal homology, as measured by meiotic pairing, has long been considered one of the fundamental features of species divergence. In most phylogenetic groups of higher organisms an increase in morphological dissimilarity is usually correlated with a reduction of chromosomal homology.

Differences in karyotypic appearance of closely related species were experimentally demonstrated to be due (at least partially) to alterations in the linear order of the chromosomal elements (Stebbins, 1950). Primarily on this basis it has been assumed that lack of (or very little) meiotic pairing in hybrids of distantly related species was due to the cumulative effects of many linear alterations (inversions and translocations). The investigations of the transfer of mosaic resistance from *Nicotiana glutinosa* to *N. tabacum* have shown that chromosomes which have almost completely lost the ability to pair may still carry equivalent genetic material (Gerstel, 1943). He clearly demonstrated that the *glutinosa Nc* chromosome could be substituted for the *H* chromosome of *tabacum*, which resulted in only slight quantitative effects although the two paired very infrequently during meiosis in hybrids.

The introgressive transfer of a genetic locus of one species into the genome of a second species involves an interspecific chromosomal exchange. The origin of such an exchange may be explained by one of two alternative hypotheses: (1) It may be a random break and reunion (a translocation). (2) The exchange may be non-random, that is, it may involve some *tabacum* chromosomes with higher frequencies than others, or it may be restricted to one or a few *tabacum* chromosomes. This could be explained as due to the presence of effective residual homology, although other mechanisms cannot be excluded, (that is, non-random translocations). The distribution of independent transfers of a single locus should be different under the two alternative hypotheses. Under the first, the introgressed locus would be distributed more or less at random in the recipient's genome, while under the second hypothesis it should be concentrated in one or a few of the recipient's chromosomes, that is, the ones that share the residual homology. The two alternative hypotheses are not mutually exclusive so it is possible that some transfers are due to random translocations

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while others are due to the presence of residual homology. In this case the distribution would remain non-random but its variance should be larger.

The locus *Ws* (*non-white seedlings*) of *N. plumbaginifolia* has been transferred repeatedly (in the present experiments) into the genome of *N. tabacum* to see whether one of the above hypotheses is more likely, and to study other features associated with interspecific gene transfer.

SPECIES USED IN THE EXPERIMENT

Nicotiana tabacum L. (subgenus *Tabacum*, section *Genuinae*, Goodspeed, 1954), n = 24, has been used as the recipient species, and *N. plumbaginifolia*, n = 10, as the donor species. Plants of *N. tabacum* (*tbc*) used in the present study were commercial types designated by growers as Red Russian tobacco (formerly variety Purpurea). *N. plumbaginifolia* (*pbg*) is referred to subgenus Petunioides, section *Alatae*, by Goodspeed. The species are widely separated on the basis of morphology, growth habit, chromosome number, and chromosome homology. Nevertheless, diploid hybrids as well as hybrid combinations with various numbers of *tbc* and *pbg* genomes may be obtained with relative ease (Cameron and Moav, 1957).

THE GENETIC MARKER USED

The occurrence of white seedlings in *N. tabacum* is determined by duplicate loci. However, Red Russian is homozygous recessive at one of these (ws_1ws_1). The Ws_2 locus has been used in the present study as the presence of recessive alleles there causes the production of seedlings devoid of chlorophyll and they may be detected immediately after germination. The dominant factor from *N. plumbaginifolia* which covers the effect of *tabacum's ws* is called *Ws* (*pbg*). It was transferred into the genome of homozygous recessive *tabacum* by appropriate crosses and selection.

EXPERIMENTAL PROCEDURES AND RESULTS

The interspecific backcross method

Because of the chromosomal sterility of F′ *tbc* × *pbg* hybrids the following scheme of crosses was used:

Step I: Autotetraploid *tbc* was crossed with diploid *pbg* (10′) to give the sesquidiploid hybrid (24″ + 10′).

Step II: The sesquidiploid (24″ + 10′) was backcrossed to diploid *tbc* (24′) and the progeny had two complete sets of *tbc* plus a variable number of *pbg* univalents (24″ + x′), when theoretically x = 0,1,2,... 10. By repeated backcrossing with automatic selection, diploid *tbc* plants possessing a single marked *pbg* chromosome were secured (24″ + 1′).

The generations following the backcross between the sesquidiploid and the diploid recipient species have been designated "Breakdown" generations, since, during this process the donor genome of the sesquidiploid is successively "broken" into independently distributed individual chromosomes. The term "Breakdown" generation is preferred over the more com-

monly used "Backcross" generation, because it includes progeny of selfed or intercrossed derivatives at any level of the breakdown process, and it excludes backcrosses which eliminate a complete genome.

Somatic instability of pbg chromosomes in hybrids and its use in the detection of introgressive chromosomal exchanges

In hybrids and hybrid derivatives of *tbc* and *pbg*, chromosomes of *pbg* were occasionally eliminated during various stages of the plant's development (Moav, 1957). When the *pbg* chromosome carrying the *Ws* (*pbg*) locus is lost in a meristematic cell of a hybrid derivative in which the *tbc* genomes possess the recessive alleles *ws*, *ws*, the cell lineage from this cell is chlorophyll deficient, which gives the plant a variegated appearance. The intensity of the variegation, as measured by the number of albino spots per leaf (SPL) was of a highly variable nature. The leaves of most hybrid derivatives of the above genotype were covered with thousands of spots. As a rule, a chromosomal interchange transferring the *Ws* (*pbg*) locus into a *tbc* chromosome resulted in an unmistakable decrease in the variegation intensity (but apparently this was not the only stabilizing mechanism). This feature provided a very convenient phenotypic criterion for the detection of most interspecific exchange products of the *Ws* (*pbg*) locus (for a detailed discussion of the somatic instability of *pbg* chromosomes see Moav, 1957).

Through elimination of the *Ws* (*pbg*) chromosome in the apical meristem of 24″ + 1 *Ws* (*pbg*) (heterozygous alien addition plants), completely albino flowers (without chlorophyll) were produced. These flowers were used in crosses where the homozygous recessive was desired as a parent.

Transmission of the alien chromosome in 24″ + 1′ types

The ovular transmission of the alien chromosome in 24″ + 1 *Ws* (*pbg*) derivatives was about 15% and its pollen transmission about 3%. Thus, the transmission on selfing was approximately 17.5% (Gerstel, 1943; Chu, 1954; Cameron and Moav, 1957).

Production of lines heterozygous for segmental substitution

Two *tbc-tbc-pbg* sesquidiploid plants possessing the recessive *ws* in both *tbc* genomes were backcrossed to heterozygous (*Ws*, *ws*) diploid *tbc*. Four mottled offspring of each cross were grown and tests showed that their *tbc* genomes possessed only recessive *ws* alleles. They did not reveal an association between the *Ws* (*pbg*) locus and the *tbc* genomes. The above eight plants were selfed and intercrossed in various ways and a total of 21,323 seedlings were germinated. These consisted of 0.95% *"Stable-Ws"* (less than 250 albino spots per leaf), 16.81% mottled plants, and 82.24% albino seedlings (table 1). Genetic tests supplemented with direct chromosomal observations of microsporocytes showed that the *Ws* (*pbg*) locus had introgressed into a *tbc* genome in about 50% of the *Stable-Ws* derivatives compared with only 4% in their mottled siblings. This provided an

TABLE 1

PROGENY SEGREGATION OF MOTTLED HETEROZYGOUS ALIEN ADDITION PLANTS OF THE FIRST BREAK-DOWN GENERATION

Parents	Progeny				% transmission of Ws, i.e. % non-albino
	Stable-Ws	Mottled	Albino	Total	
54630 p1 × s	13	82	539	1898	13.85
p3 × s	10	249	1639	634	14.98
p5 × s	18	357	1752	2129	17.69
p7 × s	51	1092	4826	5969	10.09
54631 p6 × s	34	563	2896	3493	19.14
p7 × s	11	23	203	239	14.34
p11 × s	6	101	265	372	28.76
54630 p1 × p3	4	77	435	516	15.70
p1 × p5	1	66	299	366	18.30
p1 × p7	11	152	863	1028	16.05
p3 × 631p6	3	43	228	274	16.79
p3 × 631p11	2	13	75	90	16.66
p5 × p3	3	23	202	228	11.40
p7 × p5	7	103	545	655	16.79
54631 p6 × 630p3	5	180	733	918	20.11
p7 × 630p3	2	60	372	434	14.29
p7 × 630p7	9	182	700	893	21.61
p11 × 630p3	8	124	525	657	20.09
p13 × 630p3	5	91	438	534	17.98
Totals	203		17535	21323	17.76

an easy method for production of segmental substitution lines of presumably independent origin.

Transmission rates of heterozygous segmental substitution (SS) plants

Assuming no differential reduction in zygotic viability, gametic reproductivity, and that meiotic pairing of the exchange chromosome (the one composed of *tbc* and *pbg* segments) takes place, its transmission rate should be 75%. However, as these assumptions are only very rarely valid, a lower transmission is expected, and this was actually the case in the great majority of the heterozygous-SS lines that were tested. In figure 1 the observed transmission rates of 19 heterozygous-SS lines have been plotted against the mean transmission rates of their heterozygous proggenies. The figure illustrates the wide range of parental transmission rates (30%–74%) and, most important, the high parent-offspring correlation indicated that the variability is actually due to variation in the exchange chromosome itself, rather than other external or genetic causes.

Clearly, for practical breeding, lines with the smallest disturbance (the closest to 75% transmission) are the most promising.

Male and female transmission rates

The heterozygous-SS plants were backcrossed using albino flowers that developed on mottled heterozygous addition plants (24″ + 1 (*Ws*-mott)) and

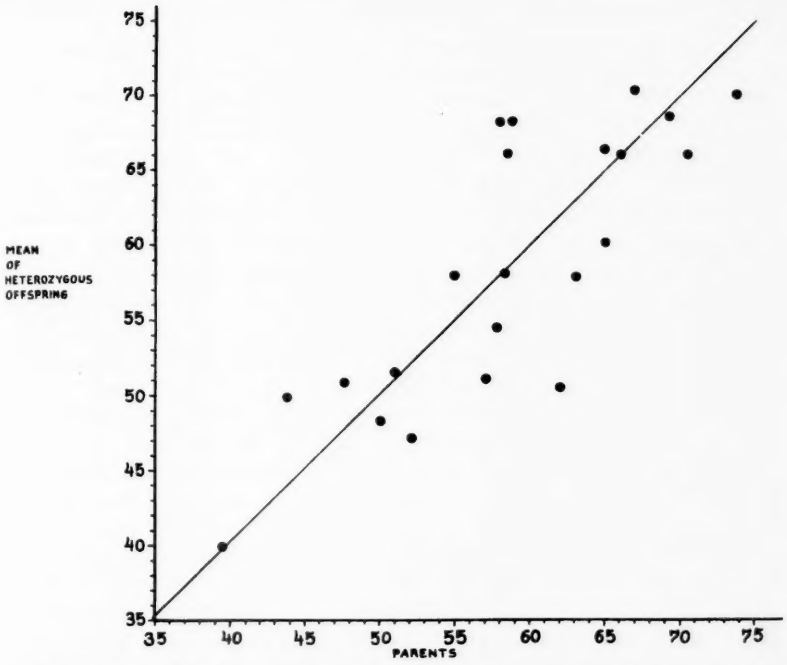

FIGURE 1. Correlation of *Ws(pbg)* transmission rates between Heterozygous-SS parents and the mean (8–14 plants) of their heterozygous offspring.

the male and female transmission rates were estimated. This also served for an estimation of the expected proportions of homozygous-SS in the offspring. The observed and calculated frequencies, which are tabulated in table 2, (only plants that were selfed and backcrossed are listed) demonstrate the following points of interest: (1) Higher variability of male transmission than of the female. (2) The mean reduction in male transmission is 18.8% (50.0–31.2), compared with 4.9% (50.0–45.1) in the female. (3) The mean "calculated" male and female transmission rates are quite close to the observed values.

The homozygous segmental substitution races

In order to produce homozygous-SS plants, progenies of 21 heterozygous-SS plants were progeny tested. The results, which are summarized in table 3, brought out several significant points: (1) of the 21 lines tested, only two had no homozygous-SS offspring. On the basis of chance alone, the probability that two or more progenies will have no homozygous-SS plants is approximately 0.44. (The observed proportion of homozygous-SS was about 50%; so the probability of a plant being homozygous-SS was taken to be 1/5. In most populations 12 plants were grown, thus the probability of

TABLE 2

OBSERVED AND CALCULATED TRANSMISSION RATES OF *Ws(pbg)* IN HETEROZYGOUS SEGMENTAL SUBSTITUTION PLANTS

	Heterozygous-SS parent	% Transmission					
		Observed			Calculated†		
		Selfed	♂*	♀	♀	♂	Self
1	54R06-2	65.0	30.5	40.9	49.6	40.8	58.9
2	R07-1	57.8	16.6	...	49.4	...	...
3	R11-1	49.9	21.4	40.6	36.1	15.7	53.3
4	R14-3	69.2	31.2	44.9	55.2	44.1	62.1
5	R19-1	58.5	22.8	...	46.2	...	...
6	R21-1	57.1	20.0	41.1	46.4	27.2	52.9
7	R21-3	70.1	44.6	52.7	46.0	36.8	73.8
8	R25-1	58.2	56.7	48.5	21.1	18.8	77.7
9	R27-1	55.0	31.1	...	34.7	...	...
10	-2	67.5	31.3	...	52.6	...	...
11	R28-3	67.4	38.6	46.9	46.9	38.6	67.4
12	R35-3	66.1	40.0	...	43.5	...	...
13	-6	65.0	40.0	...	41.7	...	...
14	-7	47.8	7.7	...	43.4	...	...
15	R36-1	67.1	30.2	...	52.9	...	...
16	-2	50.9	14.1	...	42.8	...	...
17	-3	75.7	45.5	...	55.4	...	...
18	R37-1	73.8	28.2	...	63.5	...	...
19	-2	75.0	34.1	...	62.1	...	...
20	-4	75.4	39.6	...	59.8	...	...
		63.6	31.2	45.1	47.1	31.5	63.7

*When mottled bud rather than albino was used as a tester the male transmission was corrected by subtraction of 10% from observed values.

†Formulae used for calculated values: Male $= \frac{S - ♀}{1 - ♀}$, Female $= \frac{S - ♂}{1 - ♂}$. Self $= 1 - (1 - ♀) \times (1 - ♂)$; S = selfed transmission; ♀ = female transmission; ♂ = male transmission.

TABLE 3

HOMOZYGOUS SEGMENTAL SUBSTITUTION SEGREGANTS (HOMOZYGOUS-SS) FROM HETEROZYGOUS SEGMENTAL SUBSTITUTION PARENTS

Population	Homozygous-SS (WsWs)	WsWs and Wsws	Population	Homozygous-SS (WsWs)	WsWs and Wsws
55R34	2	10	56R02	1(1)*	10
R38	4	13	R04	1(1)	10
R39	2	14	R05	4	10
R40	3	13	R07	3(2)	9
R41	6	16	R08	2(1)	10
R42	3	13	R11	2	10
R43	0	14	R12	2	10
R44	2	15	R13	2	10
R46	0	14	R14	4(2)	10
R47	2	15	R15	2	10
R48	5	15			
			Total	52(45)	251

*Parentheses represent somewhat questionable plants because about 1% of albino seedlings segregated in their offspring.

getting a progeny population with no homozygotes was $(4/5)^{12} = 0.07$. Each population is a binomial variable that may have no homozygotes with a probability of 0.07, and may have at least one homozygote with a probability of 0.93. Thus, the probability of obtaining at least two populations with no homozygotes from a total of 21 populations may be obtained from the binomial $0.07 + 0.93^{21}$. The actual probability is $1 - .93^{21} - 21 \times 0.93^{20} = 0.44$.) Thus, it can be concluded that probably all, and certainly most, of the SS lines were capable of growth and reproduction in the homozygous condition. (2) The proportion of homozygous-SS is a function of the gametic transmission of the "exchange chromosome" and the relative viability of the homozygous-SS zygotes. Since the female and male transmission rates of many of the lines were known, the expected frequency of homozygous-SS plants in the progeny could be estimated. Three semi-independent estimates were made: (1) observed ♀ × observed ♂ = 14.1%, (2) observed ♀ × calculated ♂ = 14.2%, (3) calculated ♀ × observed ♂ = 14.7%.

The agreement between the three estimates is quite close, and all were lower than the observed means (52/251 = 20.7% or 45/211 = 17.9%, table 3). This is very surprising since the viability of the homozygous-SS zygotes was expected to be reduced.

An evaluation of the morphological and physiological differences between the different homozygous-SS lines has not been attempted. However, it was obvious that variation of quantitative characters existed between most of the lines. This was expressed in depth of flower color, vigor, number of days from germination to blooming, flower and capsule size, pollen abortion, etc.

Determination of the relative position of the introgressive exchanges in the tabacum genome

The determination of the relative introgressive chromosomal exchanges of the SS lines in relation to each other was required for the testing of the present hypothesis. This has been achieved in the following simple fashion. When any pair of homozygous-SS lines is intercrossed, the progeny of the F_1 should breed true if the two lines have the same *tbc* chromosome in their "exchange chromosome pair." However, if different *tbc* chromosomes are involved, then a certain proportion of homozygous recessive progeny (albino seedlings) should segregate out according to the following scheme:

Parents $23'' + Ws^1Ws^1 \times 23'' + Ws^2Ws^2$

If the same *tbc* chromosome is present in parental lines:		If different *tbc* chromosomes are present:	
F_1	$23'' + Ws^1Ws^2$	F_1	$22'' + Ws^1Ws^0 + Ws^2Ws^0$
F_2	all green	F_2	15 green : 1 albino

The 15 : 1 ratio was derived on the assumption of no differential reproductivity of the exchange chromosomes. However, as may be seen in table 2, there was a reduction in the transmission of the exchange chromosome

TABLE 4

PERCENTAGE OF ALBINO SEEDLINGS IN F_2 OF CROSSES BETWEEN HOMOZYGOUS SEGMENTAL SUBSTITUTION LINES

♀\♂	RR*	55 R34	R38	R39	R40	R41	R42	R44	R48	56 R05	R07	R08	R11	R12	R13	R14	R15
RR*	0.0														9.4		
55R34	6.7		14.4				0.0						0.0				
R38	9.2			4.2		7.7	17.5								0.0		
R39																	
R40			15.2		0.0	1.1		0.0									
R41	0.0	2.8	8.2			0.0											
R42								0.0		0.0							
R44																	
R48	0.0			1.4				15.2							7.6		
56R05	6.1		12.4					0.0		0.0	0.0						
R07	6.3							0.0									
R07	11.6	29.1	6.1			35.0											
R11			17.4	1.9				0.0									
R12			5.7							22.7	28.0	0.0	21.6	0.0			
R13				6.1				12.0									
R14			10.0	32.0	27.2	38.2			0.0					0.0	6.9	0.0	24.0
R15		0.0	1.9						12.3	0.0					13.8		

*RR is the Variety "Red-Russian" of tabacum.

in most of the SS lines. The magnitude of the reduction which was variable between the lines, should determine the expected proportion of albino seedlings in the F_2. For instance, when two non-homologous lines with maternal transmission of 40% and paternal transmission of 30% were crossed, the expected proportion of albino seedlings was 17.64% $[(0.6 \times 0.7)^2 \times 100]$.

Zygotes containing more than one exchange chromosome are likely to be lower in viability and reproductive ability than those with a single exchange chromosome. This factor would further reduce the transmission of the introgressed locus and therefore may explain the appearance of a very high proportion of homozygous recessive plants in some of the inter-line crosses (table 4). On the other hand, the longer the *pbg* segment in the exchange chromosomes, the higher should be the expected frequency of their meiotic pairing with each other, even though they are attached to different *tbc* chromosomes. This may lower considerably the transmission of the non-introgressed homozygous recessive genotype, which might be the explanation of the very low transmission obtained in the following inter-line crosses: 55R41 × 55R34; 55R40 × 55R41; 55R48 × 55R39; 56R11 × 55R39; and 56R15 × 55R38 (table 4). Undoubtedly, other non-trivial factors also influenced the proportion of the segregating progenies.

TABLE 5

DISTRIBUTION IN THE TABACUM GENOME OF CHROMOSOMAL EXCHANGES IN 14 SEGMENTAL SUBSTITUTION LINES

	Tabacum Chromosome	I*	II	III	IV	Undetermined
1	Homozygous-SS lines	55R41	55R34	56R38	56R08	55R39
2		55R48	55R40	56R13	56R12	55R47
3			55R42			
4			55R44			56R04
5			56R05			56R14†
6			56R07			
7			56R11			
8			56R15			

*The chromosome which carries the *Ws* locus of *N. tabacum* in the "Red-Russian" variety.

†56R14 gave inconsistent results, which have not been investigated further.

The linkage relationships of the introgressed locus *Ws(pbg)* of the indedendent homozygous-SS lines, as determined by the inter-line crosses, are summarized in table 5. Its relative location in 14 lines has been determined, and they all fall into only four *tbc* chromosomes, one of which (designated I) is the *tbc* chromosome which carries the original *Ws* locus of *tbc*. This chromosome was involved in only two exchanges compared with chromosome II which has participated in eight of the fourteen exchanges. The inclusion of all fourteen exchanges in only four *tbc* chromosomes is strong experimental evidence for the non-randomness of the interspecific exchange. The presence of residual homology seems to the present writer

as the most likely causal mechanism, although other hypotheses, that is, non-random translocations cannot be excluded.

DISCUSSION

The plant breeder needs a large gene pool to supply him with desired properties for the genetic improvement of his stock. Clearly, the larger the store of genes which could be transferred by synthetic introgression (artificial gene transfer) into the commercial variety, the larger is the range of potential genetic improvement. Whenever the desirable genes are found in related varieties or closely related species which behave as varieties in hybridization (no hybrid sterility and inviability, etc.), the job is relatively easy. One need only to hybridize and, by recurrent backcrosses and selection, the desirable *introgressed* or *backcross* derivatives may be obtained. This method has been used frequently in crop improvement (Briggs, 1935; Thomas, 1952). When desirable genes are found in distantly related species, reduced chromosomal homology is one of the most important obstacles because it decreases chromosomal exchange. Frequently, chromosomal sterility may be overcome by the production of amphidiploid and sesquidiploid hybrids (Gerstel, 1943). The partially fertile sesquidiploid may be backcrossed to the recipient species and a product obtained with a normal complement plus a single donor chromosome. By selfing or backcrossing of the alien addition plants three classes of true breeding hybrid derivatives may be obtained: (1) *Homozygous addition races* in which a complete pair of chromosomes has been added. (2) *Homozygous substitution races*, where a complete pair of the recipient's chromosomes has been replaced by a pair of the donor's chromosomes. (3) *Homozygous segmental substitution races*, where, through interspecific chromosomal exchange, the donor chromosomal segment carrying the desirable locus has replaced only a segment of a recipient chromosome. The first alternative is usually of little or no value to the breeder, because it introduces too many undesirable genes in addition to the one under selection. In the homozygous substitution types the replacement of a whole chromosome pair usually proves too deleterious. This leaves only homozygous segmental substitution as the introgression product of practical importance.

Independently introgressed lines of the same donor locus in the same recipient species were not uniform. In general, given an equivalent chromosomal background, four parameters are responsible for the variation among the various homozygous-SS lines for the same locus: (1) The specific *tbc* chromosome to which the *pbg* segment has been attached. (2) The length of the missing *tbc* segment. (3) The specific point of attachment on the *tbc* chromosome. (4) The length of the incorporated *pbg* segment.

The finding that homozygous-SS lines of the same locus are not uniform indicates that failure of a single homozygous-SS line to meet commercial requirements must not lead to adverse judgment concerning the value of any particular gene transfer. *The breeder's goal is the smallest possible segment in the position of least disturbance.* He must produce as many inde-

pendent SS lines as practically possible to ensure a larger coverage of the SS range. From this range he may select the line which would best serve his improvement requirements.

The subjection of the heterozygous alien addition plants (one extra alien chromosome) to translocation-producing mutagens is an alternative method for the transfer of a locus from one species to another. Mutagenic agents may increase the rate of introgressive chromosomal exchange (a limiting factor when the "natural" method is used). This method has been used successfully by Sears (1956). The present experiments demonstrate that the transfer of the marker under investigation (*Ws* of *N. plumbaginifolia*) was non-random (table 5). Rare interspecific meiotic pairing and crossing over in the hybrid meiocytes due to the presence of *residual homology* between the chromosomes of the two species is a likely explanation for the strongly selective chromosomal exchange. This explanation also agrees with our previous knowledge of chromosomal behavior in interspecific hybrids. The sharing of residual homology between a single *pbg* and several *tbc* chromosomes may be easily explained as due to the establishment of different translocations in the genomes of the two species during their evolutionary history.

Acceptance of the role of residual homology in interspecific chromosomal exchange of the alien addition plants raises a serious theoretical objection to the use of mutagens for the above purpose. A mutagen-induced translocation is a random event in the sense that it may cause a break and union in any of the recipient's chromosomes and at many positions of each chromosome. The random translocations do not utilize the presence of residual homologies which are used by the "natural" process. A random exchange would replace most of the eliminated recipient's genes with ones controlling entirely different metabolic functions, and duplications which are apt to cause an unbalance of other physiological processes. On the other hand, when residual homology is utilized, the chances are much better that at least in some of the possible SS lines the introgressed segment would substitute successfully for most of the replaced genes.

SUMMARY

In alien addition hybrid derivatives (two recipient genomes plus a single alien chromosome) of distantly related species, occasionally the alien chromosome will exchange segments with a member of the recipient's genome. The exchange may be explained by either or both of two hypotheses: (1) it may be a chance break and reunion (a translocation) in the recipient's genome or (2) it may be due to crossing-over between two segments with rarely effective *residual homology*.

In order to empirically test the alternatives the following experiments were conducted:

1. A single dominant genetic marker, *white-seedling* (*Ws*) of the species *Nicotiana plumbaginifolia* has been transferred repeatedly into the genome of *N. tabacum* by use of the interspecific backcross method.

2. Presumably independent homozygous segmental substitution lines (19) have been produced by selfing four independently originated heterozygous segmental substitution plants.

3. Variability of a quantitative nature among the homozygous segmental substitution lines, was observed.

4. Determination of the *tbc* chromosomes involved in the incorporation of the *pbg* chromosome has shown that out of 14 transfers eight involved the same *tbc* chromosome. The remaining six were equally distributed among three other *tbc* chromosomes. This distribution is clearly non-random, and therefore supports the hypothesis that residual homology existed between the two species and was responsible for the interspecific exchanges.

The use of mutagenic agents to increase the rate of interspecific chromosomal exchange is discussed in the light of the present findings.

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LITERATURE CITED

Briggs, F. N., 1935, The backcross method in plant breeding. J. Amer. Soc. Agron. 27: 971–973.

Cameron, D. R. and Rom Moav, 1957, Inheritance in Nicotiana tabacum XXVII. Pollen killer, an alien genetic locus inducing abortion of microspores not carrying it. Genetics 42: 326–335.

Chu, H. Y., 1951, The mechanism of transfer of a gene from *Nicotiana glutinosa* to *N. tabacum.* Unpublished Ph.D. thesis, deposited in Univ. of Calif. Library.

Clausen, R. E., 1952, The cytogenetics of introgression. Science 115: 481.

Clausen, R. E. and D. R. Cameron, 1950, Inheritance in Nicotiana tabacum XXIII: Duplicate factors for chlorophyll production. Genetics 35: 4–10.

Gerstel, D., 1943, Idem. XVII: Cytogenetical analysis of glutinosa-type resistance to mosaic disease. Genetics 28: 533–536.

1945, Idem. XX: The addition of N. glutinosa chromosomes to tabacum. Jour. Hered. 30: 197–206.

1948, Transfer of the Mosaic-Resistance factor between H chromosomes of *Nicotiana glutinosa* and *N. tabacum.* Jour. Agr. Res. 76: 421–427.

Goodspeed, T. H., 1954, The Genus Nicotiana. Chronica Botanica Co., Waltham, Mass.

Moav, Rom, 1957, Somatic chromosome instability in Nicotiana hybrids. Unpublished Ph.D. thesis, deposited in Univ. of Calif. Library.

Sears, E. R., 1956, The transfer of leaf rust resistance from *Aegilops umbellulata* to wheat. Genetics in Plant Breeding. Brookhaven Symposia in Biology 9: 1–22.

Stebbins, G. L., 1950, Variation and evolution in plants. Columbia Univ. Press, New York, N. Y.

Thomas, M., 1952, Back-crossing. Tech. Communication 16. Commonwealth Bureau of Plant Breeding and Genetics, Cambridge, England.

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FOOD PREFERENCES OF DROSOPHILA LARVAE*

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In their natural environment Drosophila feed on yeasts, molds, and bacteria, but relatively little is known about their food requirements and feeding habits. Wagner (1944) found that eight yeasts isolated from cactus fruit permitted complete development of *D. mulleri* larvae, but only five of these yeasts supported complete development of *D. aldrichi*, indicating a difference in the ability of these species to utilize various foods. Dobzhansky and Pavan (1950) reported that a species might be found on a particular fruit in one locality and not in another, and they therefore suggested that the differences in microflora were responsible for the distribution of various species on these fruits. Differential attractiveness of various species of yeasts to different species of Drosophila has been demonstrated by Buzzati-Traverso (1949) and DaCunha, Dobzhansky and Sokoloff (1951). Carson et al (1956) and Phaff et al (1956) isolated yeasts from the crops of adult Drosophila and from the few known breeding sites of the flies, oak slime fluxes. They found a striking difference between the two florae, and Phaff suggested that these differences may diminish the competition for food between the larval and adult forms of the same species.

It would be interesting to determine whether Drosophila larvae exhibit any preference for a particular type of food. If not, the composition of their diets will be a function of the relative abundance of various foods dependent upon the choice of egg laying sites selected by the parent fly. The present study was undertaken to determine whether Drosophila larvae can distinguish between species of yeasts offered to them by showing preferences for particular types of yeast; whether there are differences in the pattern of food preferences of different species; and finally, of what value these yeast types may be to the development and survival of Drosophila.

MATERIAL AND RESULTS

Four types of yeasts, *Saccharomyces montanus*, *S. veronae*, *S. ceriviseae* var. *tetrasporous*, and *S. drosophilarum*, were isolated from the crops of *D. pseudoobscura* adults collected in the Mather area by Phaff (1956). Stocks of these yeasts obtained from Dr. Phaff have been used in the present study. These stocks were maintained on Difco Nitrogen-Base yeast medium containing 0.5 per cent dextrose and 1.5 per cent agar. The following abbreviations will be used throughout the remainder of this discussion: *S. montanus* = *M*; *S. veronae* = *V*; *S. ceriviseae* = *C*; *S. drosophilarum* = *D*.

*This paper is a summary of a thesis presented for the B.A. degree from Reed College.

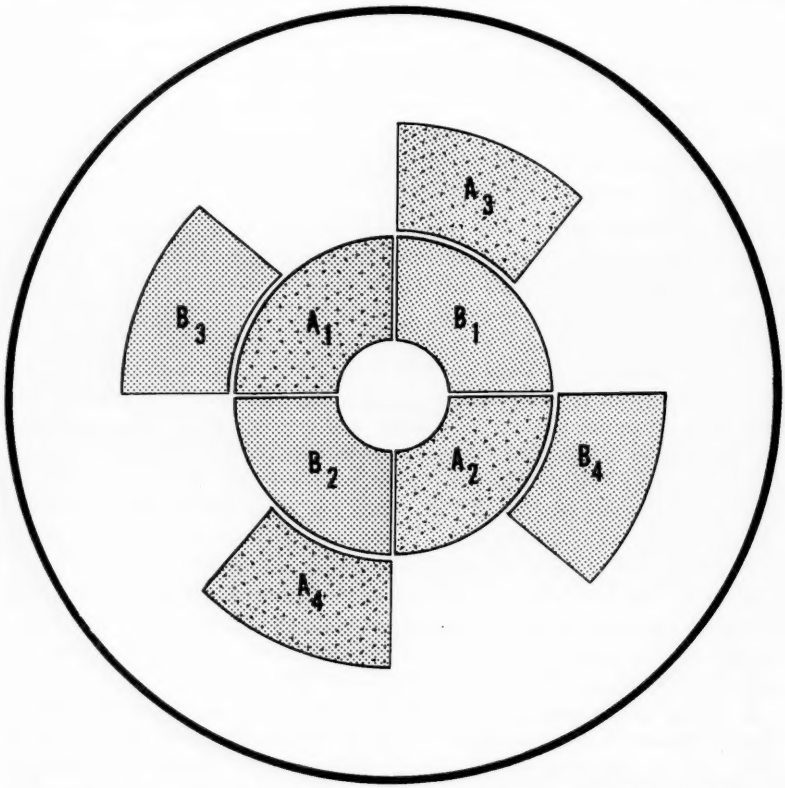

FIGURE 1. The pattern in which yeasts were grown on petri dishes. In experimental plates all A sections contained the same yeast type and all B sections were plated with the other yeast type. Control plates had one type of yeast plated on all eight sections.

Eggs of *D. persimilis* (delta glaze) and *D. pseudoobscura* (Standard) were collected on paper spoons containing cream of wheat-molasses medium (Spassky, 1943) coated with a mixture of yeast and honey. Flies were allowed to deposit eggs for 24 hours on spoons which were then stored in an incubator at 27 ± 1°C for another period of 24 hours. The larvae which had hatched at this time were then transferred with a dissecting needle to stender dishes. Twenty-five larvae were grown in each stender dish on molasses-cream of wheat medium with Fleischmann's yeast for 48 hours at 27 ± 1°C. The larvae were then removed from the stender dishes, washed in Drosophila Ringer solution, and placed in petri dishes which had previously been plated with yeast. The technique of replica plating described by Lederberg and Lederberg (1951) has been utilized to prepare the petri dishes with yeast. A master plate was made by painting the yeast suspension in a definite pattern on the plate with a cotton swab (figure 1). These

master plates were grown at 27 ± 1°C for 48 hours, and then five copies of each plate were made. The replicated plates were incubated at 27 ± 1°C for 24 hours at which time ten larvae were released at the center point of each petri dish. Five experimental plates and five control plates were used for each experiment; control plates had one type of yeast on all eight sectors while experimental plates had two types of yeast. The larvae were left on the plates for about three hours and then the number of larvae on each section of yeast was counted and recorded. Only actively feeding larvae were taken into consideration when scoring the distribution of larvae in the different segments of yeasts in the experimental and control plates. The larvae which had burrowed under the medium or crawled in cracks were disregarded because the distribution of these larvae could be due to thigmotropic responses rather than to preference for any type of yeast.

The results of the experiments on food preference were evaluated by comparing the total number of larvae found on the A sections of the plates with the total number found on the B sections. Assuming that there are no preferences, larvae are likely to be evenly distributed in all sections of A and B and a 1 : 1 distribution of larvae in these segments is expected. The χ^2 values for the control plates do not deviate significantly from a 1 : 1 ratio whereas there are significant deviations from the expected in many of the experimental plates (see table 1). To determine whether any unknown factors such as light and temperature variations may have affected the distribution of larvae, the number of individuals in each of the four quadrants of

TABLE 1

DISTRIBUTION OF LARVAE ON PLATES WITH TWO YEAST TYPES AND CONTROL PLATES

Experimental plates			Control plates		
Yeasts	No. larvae on each yeast	χ^2	Plate sections	No. larvae on each	χ^2
D. pseudoobscura					
C–M	66–77	0.78	A–B	71–63	0.48
V–M	87–45	13.30*	A–B	63–65	0.03
D–M	78–72	0.24	A–B	71–51	3.28
V–C	87–51	9.40*	A–B	54–45	0.82
C–D	55–74	2.79	A–B	55–62	0.41
V–D	70–78	0.43	A–B	56–56	0.00
D. persimilis					
C–M	75–27	22.60*	A–B	33–43	1.31
V–M	48–42	0.40	A–B	33–39	0.50
D–M	52–45	0.50	A–B	44–45	0.01
V–C	20–64	21.90*	A–B	44–29	3.06
C–D	41–72	8.50*	A–B	61–54	0.42
V–D	25–72	22.80*	A–B	47–40	0.56

The total number of larvae for each combination is the sum of four experimental runs. Those experiments which show a deviation from a 1 : 1 ratio as shown by χ_1^2 d.f. are designated with an *.

control plates was compared. Assuming an equal distribution in all quadrants, the data were tested for an expected 1 : 1 : 1 : 1 ratio. None of the χ^2 values were significant, hence there is no reason to suspect any appreciable bias in the distribution of larvae by these unknown factors in these experiments.

Before pooling the data from different plates, inter-plate reproducibility was examined by using the χ^2 test for homogeneity for each group of experimental and control plates. The results within each group were found to be homogeneous. *D. pseudoobscura* larvae showed a deviation from random distribution in these combinations of yeasts: *V*-*M* and *V*-*C*, in both cases *V* was preferred (table 1). In the case of *D. persimilis* a deviation from a 1 : 1

TABLE 2

PREFERENCE INDICES FOR YEAST TYPES AND t-VALUES FOR COMBINATIONS OF YEASTS

Yeast	N	Index	σ	t-values for combinations		
				C	M	D
D. pseudoobscura						
S. veronae	418	0.584	0.0241	4.81*	3.76*	1.29
S. drosophilarum	427	0.540	0.0241	3.50*	2.46*	
S. montanus	425	0.456	0.0242	1.08		
S. ceriviseae	410	0.419	0.0244			
D. persimilis						
S. veronae	271	0.344	0.0289	5.29*	1.23	7.38*
S. drosophilarum	307	0.638	0.0274	1.43	6.14*	
S. montanus	289	0.394	0.0287	4.68*		
S. ceriviseae	309	0.582	0.0281			

The t-values which show a significant difference between the preference indices of combinations of two yeasts are indicated by an *. N is the total number of larvae recorded in all experiments involving a given yeast type.

ratio was shown in the combinations *D*-*V*, *D*-*C*, *C*-*V*, and *C*-*M*. *D* was preferred to *V* and *C*; *C* was preferred to *V* and *M*. On the basis of the data reported, an arbitrary index of preference was obtained in the following manner. The yeast type *C* was tried in three combinations: *C*-*D*, *C*-*M*, *C*-*V*. The total number of larvae scored in each experiment involving *C* was obtained and the percentage of larvae found on *C* was taken as the arbitrary index preference for *C*. In a similar manner the preference index for each yeast with respect to the other three yeasts was determined and is shown in table 2. *D. persimilis* showed the greatest preference for *D*, followed in order by *C*, *M*, and *V*; on the other hand *D. pseudoobscura* showed the greatest preference for *V* followed by *D*, *M*, and *C*. The differences in percentages were evaluated by using the t-test (see table 2). In the case of *D. pseudoobscura* there was a significant difference in the preference indices for *C* vs *V*, *D* vs *M*, *C* vs *D*, and *M* vs *V*. For *D. persimilis* these were different for *C* vs *V*, *C* vs *M*, *D* vs *V*, and *M* vs *D*.

The percentage survival of *D. pseudoobscura* (Standard) and the developmental time on each of the four yeasts was examined in the following manner. Four sets of one-half ounce creamers containing cream of wheat-molasses medium were inoculated, each with a thin suspension of one of the four yeasts. These creamers were kept at 24 ± 1°C for 36 hours. Newly hatched larvae were collected from unyeasted spoons containing cream of wheat-molasses medium, and ten larvae were placed in each creamer. The creamers were then incubated at 24 ± 1°C and the number of adults which hatched in each creamer recorded daily beginning on the 14th day. The percentage of larvae of *D. pseudoobscura* surviving to the adult stage on each of the four types of yeast was the same (about 85 per cent). An analysis of variance showed that there was no more variation in per cent survival between the four groups than there was within each group. There was, however, a definite difference in time of hatching. On the basis of 75 per cent emergence of adults, there was an average difference of one day hatching time between *C-D*, and *D-M* and a half day difference between *M-V*. This difference in hatching time may be due to differences in the growth rates of the yeasts, or it may be a function of the nutritive value supplied by each yeast type.

DISCUSSION

The maintenance of chromosomal or genetic polymorphism in Drosophila can be due to many different causes. One of the simplest mechanisms would be the superiority of heterozygotes over homozygotes insuring the presence of homozygotes in the population (Wright and Dobzhansky, 1945). Certain homozygotes are better adapted to the ecological conditions prevailing at one time of the year than at another. Dobzhansky (1943) and Wright and Dobzhansky (1945) demonstrated that the frequencies of certain chromosomal types of *D. pseudoobscura* undergo seasonal changes in a cyclic manner. The existence of a number of food niches in the environment can maintain genetic polymorphism within a population since some genotypes have higher adaptive values than other genotypes in relation to the same food niches. DaCunha et al (1951) were able to demonstrate experimentally that different genotypes of *D. pseudoobscura* varied in their adaptive values on different foods.

If adaptive gene complexes are constantly selected for a particular food niche in the environment, it is conceivable that a correlated selection for food discriminatory behavior on the part of the individual may also take place. This will mean that the individuals of a population will actively seek the food to which their genotypes are best adapted. Food preferences of adult Drosophila have been examined (Buzzati-Traverso, 1949; DaCunha et al, 1951; Shihata et al, 1953; Phaff et al, 1956), and both in nature and in laboratory experiments Drosophila adults were differentially attracted to yeast types. The results of the present experiments suggest that the larvae of Drosophila also show a preferential distribution in yeast choice experiments. The larvae may be able to distinguish between the yeasts by a trial

and error method of eating. It is also possible that the yeasts produce groups of diffusible substances to which the chemoreceptors of the larvae are sensitive and which could attract larvae across a distance on the agar plates. It is interesting that the larvae discriminate between some yeasts, but do not distinguish between their most and least preferred foods when these are presented in combination. The following is an example of this situation as found in *D. persimilis*. *D* was preferred to *C* and *V*; *C* was preferred to *V*. But in both the *D*-*M* combination and the *V*-*M* combination no preferences were shown. *C* was preferred to *M*. This situation may be represented as follows:

$$\begin{array}{ccccc} D & > & C & > & V \\ & \diagdown & \vee & \diagup & \\ & & M & & \end{array}$$

These results indicate that the factors affecting food choice are very complex.

Phaff et al (1956) and Carson et al (1956) have surveyed the distribution of yeasts in the crops of adult *D. pseudoobscura* and *D. persimilis*, and other species, as well as the yeast flora of the slime fluxes where these flies breed and their larvae grow. There was a striking difference between the two yeast flora. The yeasts isolated from the adult flies' crops were largely fermentative types, whereas the yeasts from the slime fluxes were primarily non-fermentative. These authors suggested that the differences between the two florae can ease the intraspecific competition between larvae and adults. It remains puzzling that the yeasts from one source are different than those from another. Does this mean that the adults select one food type for themselves and a different type for the next generation larvae? The mobility of Drosophila larvae is more restricted than that of the adult flies. The larval stage is a period of high feeding activity, and the choice of egg laying sites made by the adult female will determine the location of the feeding activity of the larvae, and their approximate pupation site. Our preliminary experiments indicate that the larvae of Drosophila do have the ability to make a choice of what they will eat. Whether or not choice is available to the larvae in a natural state remains to be investigated. The observations on yeast flora by Phaff and Carson have been confined to the adult males of the species. Since the female selects the breeding site, it would be interesting to compare the yeast flora collected from gravid females with yeast preferences of larvae.

All four yeasts used in the present experiments support the complete growth of *D. pseudoobscura*. There is, however, a difference in the hatching time of the flies raised on these yeasts. It is interesting to note that the yeast *S. ceriviseae* which gave the shortest hatching time was the type which the larvae preferred least. And vice versa, *S. veronae* which results in a longer growth and hatching period was the type chosen by the larvae. These results may indicate the potentiality of the genotype in relation to the yeast type. Under certain conditions the Standard genotype competing

with other genotypes will be at an advantage when the larvae are raised on yeast which accelerates hatching time. Slow hatching may also be advantageous in the winter and spring when the weather is cold and food scarce.

SUMMARY

A method for determining food preferences in Drosophila larvae was developed to test the preferences of *D. pseudoobscura* and *D. persimilis* larvae. Both were found to show preferences among four species of yeast which they commonly eat, and these preferences differed markedly between the two species. The per cent survival and the hatching time of *D. pseudoobscura* on the same four yeasts were determined. There was no difference in the per cent survival, but there was a difference of two and a half days in the average hatching time.

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LITERATURE CITED

Buzzati-Traverso, A., 1949, Preference of *Drosophila subobscura* for wild yeasts. Drosophila Information Service 23: 88.

Carson, H. L., E. P. Knapp, and H. J. Phaff, 1956, Studies on the ecology of Drosophila in the Yosemite Region of California, III. Ecology 37: 538–544.

DaCunha, A. B., Th. Dobzhansky, and A. Sokoloff, 1951, On food preferences of sympatric species of Drosophila. Evolution 5: 97–101.

Dobzhansky, Th., 1943, Genetics of natural populations, IX. Genetics 28: 162–186.

Dobzhansky, Th., and C. Pavan, 1950, Local and seasonal variations in relative frequencies of species of Drosophila in Brazil. J. Animal Ecology 19: 1–14.

Lederberg, J., and E. M. Lederberg, 1951, Replica plating and indirect selection of bacterial mutants. J. Bact. 62: 399–406.

Phaff, H. J., M. W. Miller, J. A. Recca, M. Shifrine, and E. M. Mrak, 1956, Studies of the ecology of Drosophila in the Yosemite Region of California, II. Ecology 37: 533–538.

Shihata, A. M., El Tabey, and E. M. Mrak, [illegible]3, Intestinal yeast flora of successive populations of Drosophila. Evolution 6: 325–332.

Spassky, B., 1943, Cream of wheat-molasses fly medium. Drosophila Information Service 17: 67–68.

Wagner, R. P., 1944, The nutrition of *Drosophila mulleri* and *Drosophila aldrichi*. Growth of the larvae on cactus extract and the microorganisms found in cactus. Univ. of Texas Publ. No. 4445: 104–128.

Wright, S., and Th. Dobzhansky, 1945, Genetics of natural populations, XII. Genetics 31: 125–156.

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OBSERVATIONS ON SOME IMMUNE REACTIONS OF THE SIPUNCULID WORM DENDROSTOMUM ZOSTERICOLUM*

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The present paper gives the results of some experiments investigating the responses of the sipunculid worm *Dendrostomum zostericolum* (Chamberlain) to materials placed in its coelom. These experiments were done for the purpose of determining whether or not tissue antagonisms and other immune reactions, comparable to those in vertebrates, could be detected in this species. The relation of our observations to the study of antibody production by invertebrates is considered in the discussion.

MATERIALS AND METHODS

The sipunculid worm *D. zostericolum* was selected as a suitable invertebrate animal for these studies for several reasons. Notably, it was readily available, lived well in the laboratory and was of convenient size and morphology. As described in Fisher (1952), these worms are of relatively simple structure. The most attractive feature for immunological research is that internally they resemble a large tough walled sausage-shaped bag of coelomic fluid, with contractile tentacles at one end, in which the intestines and other organs are suspended. The fluid is isotonic with sea water and contains a large number of hemerythrocytes, various white cells, here termed hemocytes, and sperm or eggs depending on the sex. Coelomic fluid can easily be injected or withdrawn from the worms using a 24 or 26 gauge needle, a 10 mm. animal safely yielding about 1 ml. The fluid does not clot readily and lends itself to a variety of experiments.

Animals were narcotized before operating, since upon handling they contract very tightly, becoming turgid and withdrawing their tentacles. Ten per cent ethanol in filtered sea water was used, following the technique of Peebles and Fox (1933). This reagent caused complete relaxation of most animals within a period of three minutes to an hour. Higher concentrations of ethanol caused bleeding and untimate degeneration of the tentacles.

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Chloretone was tried, but was found to be lethal at concentrations of one part per thousand and ineffective at one part per five thousand. Tricaine methanesulfonate (MS222), at a concentration of one part per thousand, caused relaxation only after about 17 hours.

Worms were kept either in sand in running sea water, or in individual 400 ml. beakers containing 0.008M sulfathiozole in sea water. This medium was changed every two or three days and was kept at a temperature of 14-16°C. The sulfathiozole was used to reduce the chance of infection that sometimes occurred following operations. The worms in the beakers appeared to feed on materials in the sea water, and excreted fecal material during the course of the experiments.

Peebles and Fox in their extensive studies on this species have shown that *D. zostericolum* is "exceptionally viable and adaptable to radically different environments," living for a week or more in media from which the oxygen has been exhausted, and for months in stagnant sea water. With this the case it is probable that no differences in the specificity of the reactions observed would have been found by placing worms in environments other than those studied. However, the main conclusions reached in this paper are based on observations made on animals kept under sand in running sea water, supplemented by additional data from experiments made on beaker-contained worms.

SKIN TRANSPLANTS

Experiments with skin transplants on beaker-contained animals contributed no data concerning immune responses since certain difficulties were not overcome. The results are given below in order to show the nature of the work performed. Squares of epidermis and overlaying cuticle, about one square centimeter in area, were reciprocally exchanged between pairs of animals kept individually in beakers. As a control each animal was given an autograft by removing a square of epidermis and cuticle, of a size equivalent to that of the homograft, and then replacing this square in its original position. The autografts and homografts were always placed directly adjacent to each other. It was necessary, while preparing the graft, to exercise great care in keeping the underlying muscle layers intact since the slightest damage to these tissues almost invariably caused a rupture of the body wall either at the time of the operation or one to four days later.

Grafts were at first sutured into place until it was found that unsutured portions raised from the underlying tissue bed. They were then applied without sutures by sponging the wound area so as to present a sticky surface, and leaving the worm out of water for a short time before placing the graft. All air bubbles beneath the graft were removed by gently massaging the graft area with a dull probe. Twenty-six animals treated in this manner retained both auto- and homografts for periods of from eight to fifteen days. However, in spite of the prolonged contact between graft and host no heal-

ing was observed and both kinds of grafts eventually fell off without revealing any selective affinities between the tissues concerned. Following these observations attention was directed to the transplantation of tentacles.

TENTACLE TRANSPLANTS

A variety of observations were made upon tentacle transplants. These utilized the basic discovery that materials inserted into the coelom of *D. zostericolum* are encapsulated by the hemocytes. Such encapsulation appears to be a common type of immune response among the invertebrates, (Cf. Cameron 1932, and Salt, 1957). The operation involved consisted of placing bits of severed tentacles either partially or completely in the coelomic cavity of worms. Sometimes these were held in place by surgical silk and other times left free, the slit through which they were introduced being sutured shut. Excessive loss of fluid was avoided by removing about a milliliter of fluid and returning this to the coelom immediately following the operation. Worms operated on in this manner apparently kept healthy in running sea water and also in beakers containing sulfathiozole-sea

TABLE 1

Time in coelom	Type implant	Sex	Number of worms	Degree of encapsulation					
								Averages	
				0	1	2	3	Separate	Total
4 hours	auto	♂	7		2	3	2	2.0	
	auto	♀	10		3	7		1.7	1.8
	homo	♂	8		5	2	1	1.5	
	homo	♀	7		2	5		1.6	1.6
24 hours	auto	♂	12	1	1	4	6	2.3	
	auto	♀	10	1	4	2	3	1.7	2.0
	homo	♂	14	2	1	4	7	2.1	
	homo	♀	8		2		6	2.5	2.3
5 days	auto	♂	8			2	6	2.8	
	auto	♀	12		2	3	7	2.4	2.6
	homo	♂	11		3	3	5	2.2	
	homo	♀	8		1	3	4	2.6	2.3

This table shows that sipunculids encapsulate both auto and homo coelomic transplants of tentacles at the same rate. It also shows that no difference exists between the encapsulation responses of different sexes. The rate of encapsulation was determined by assigning numbers indicating relative completeness of the capsule around any one tentacle. Numbers range from 0 (no capsule) to 3 (heavy complete capsule).

water. The highly muscular and vascular tentacles could easily be identified inside the collom as alive by observing their general motility and that of their cilia.

Table 1 summarizes the results of a series of experiments that shows that individuals of *D. zostericolum* do not distinguish between implants of tentacles from themselves as compared with those of other worms. This table also shows that male and female worms react to implants in a similar manner. (The sex of worms can be determined by observing whether their coelomic fluid contains sperm or eggs). Implanted tentacles were found in all cases to be completely viable following release from their capsules.

These results are supported by extensive series of experiments on worms kept in beakers as described in materials and methods. Worms so kept were slower to form capsules than those in running sea water. However, no differential specificities were found that involved either auto and homo transplants, or the reactions of different sexes. Encapsulated tentacles were remarkably viable, living for as long as 70 days. Severed tentacles maintained alone were also quite viable, but died on the average within 28 days.

TABLE 2

	Number of worms	Degree of encapsulation				
		0	1	2	3	Average
Pre-injected	15	2	9	3	1	1.2
Control	17			4	13	2.8

This table compares the degree of encapsulation of tentacle transplants that were made into a series of worms that had received a first transplant five days earlier, and a control series that had received no transplants. The amount of capsule formation is scored as in table 1.

The relationships between a first and second series of transplants were investigated. Two sets of worms were in these series. Individuals in one of these received a homograft in the form of about 0.1 gm. of finely chopped tentacle, individuals in the other such series received no graft. Five days later, members of each series all received homografts in the form of an intact tentacle. These worms were all opened twenty-four hours later. As table 2 shows, the worms that received only the second graft were much more thorough in encapsulating it than were those that received two grafts. This difference appears to be the result of failure to proliferate cells to replace those used in encapsulating first grafts, all pieces of which were heavily enclosed. The results strongly suggest that *D. zostericolum* has no compensatory mechanism for rapid (five days at least), replacement of such cells. The data in table 2 are supported by results from exploratory

observations on three other comparable series of worms and are in agreement with the observations of Salt (1957) on the encapsulation of the eggs of parasites in the coelom of insects. Loeb's (1945) review of transplantation in Annelida also suggests that homograft specificity does not exist in this relative of the sipunculids.

The structure of the capsules was studied microscopically. In this series as in others it is similar in some respects to the ones Cameron (1932) described as produced by the earthworm *Lumbricus*. The cells comprising the capsule present a layered effect with a group of compacted cells alternating with a layer of dense, sparsely celled fibrous tissue. Eggs or sperm and red and white cells are sparsely scattered through the substance of the capsule, and these types of cells are also found floating within the capsule in the fluid bathing the implant. Although the capsule was never fused to the implant where the implant had intact epidermis, it was, in all cases observed, tightly fused to the cut stump of the implant; so tightly fused in fact that, at this point, the stump and capsule appear to be one continuous structure.

Various other materials inserted into the coelom of *D. zostericolum* were observed to be encapsulated. These included small pieces of cellulose sponge (six worms), filter paper (four worms), plastic tubing (four worms) and sea anemone tentacles (five worms). These last were particularly interesting. Each worm that received a tentacle of the anemone *Anthopleura xanthogrammica* was opened five days later. All tentacles were heavily encapsulated, dead, and almost completely digested. In contrast, explanted anemone tentacles were still alive and mobile.

PHAGOCYTOSIS

As noted above the coelomic fluid of *D. zostericolum* contains, in addition to red cells, large numbers of white cells. Eosinophil-like cells were a very characteristic component of this population, together with various other types difficult to classify with certainty. ("Urns" are not present in this species.) The eosinophils and some of these others were amoeboid and phagocytic, injesting and digesting red cells, bacteria, and yeast, injected into the coelom. Phagocytosis could also be demonstrated in vitro quite readily. A great deal of effort was made to devise a quantitative method to study this phenomenon, but the tendency of the white cells to stick together in the presence of particulate matter prevented this from being successful. As far as can be estimated there appears to be no increase in phagocytosis or in phagocytes during a course of immunizations, a point supported by the data on tentacle transplants. However, there seems no doubt even in the absence of quantitative data that phagocytosis, as in other invertebrates, plays an important role in immunity, cellular material being removed from the coelom by phagocytes within a matter of hours.

ANTIBODY SYNTHESIS

Repeated efforts were made to demonstrate the appearance of specific agglutinins, lysins and precipitins following the injection of antigen. None of these were successful. While it is obviously dangerous to form definite conclusions from such negative data, our efforts were extensive and it seems probable that *D. zostericola* lacks the capacity for synthesizing antibody-like substances in response to specific antigens. Natural agglutinins for various sorts of red cells were observed that accented the need for careful controls, particularly as the occurrence of these was sometimes found to vary in the same animal at different times in the absence of known experimental manipulation.

Mammalian hemoglobin and chicken ovalbumin persisted for several weeks in the coelomic fluid of worms kept in beakers. This is interesting when considered in relation to the correlation between digestibility and antigenicity discussed by Campbell (1957) and is a subject that is to be further investigated.

The question has been raised at various times as to whether or not specific antibodies can be synthesized by any organisms other than vertebrates. Present evidence (reviewed in Cushing and Campbell, 1957) is inadequate to answer this question. This leaves the intriguing possibility open that the mechanism of antibody synthesis may have evolved as a peculiar property of the vertebrates. The study of this point assumes particular significance when it is realized that antibodies are the only proteins known that appear to be synthesized under the guidance of both genetic and environmental influences. This point arises from the consideration that while the synthesis of the combining sites of antibodies is directed by specific antigen, their antigenic properties are determined by the genetic constitution of the species in which they occur.

As further investigation of the evolution of antibody synthesis holds considerable interest, a search was made for animals suitable for this purpose. Observations at the Marine Biological Laboratory, Woods Hole have shown that the pecten *Pecten irradians* is a particularly promising animal with regard to tissue transplantation (Cushing, 1957). In addition, the sea cucumber Thyone seems to offer some promise for transplantation work as it can be anesthetized with chlorotone, and the coelomic fluid contains a variety of cells, some types of which are actively involved in excretory functions. These include the encapsulation of foreign materials (cf. the *brown bodies* referred to in Hyman, 1955). Spider crabs were found to be easy to bleed and inject, and to remain in good condition and feed in the laboratory. Additional papers have been assembled in the literature cited which are of interest in considering the experimental study of antibody production by invertebrates. Among these are those that describe the natural heterotransplantations made by nudibranchs as they feed upon anemone tentacles and transfer their nematocysts into their own bodies (cf. Glaser, 1910 and Kepner, 1943).

SUMMARY

The reactions of sipunculid worms to auto and homo coelomic transplants of tentacles were investigated. These transplants were found to be encapsulated by host hemocytes in the coelomic fluid at rates independent of their origins. No enhancement of phagocytic numbers or encapsulation rate was observed in second series transplants. No antibody production could be demonstrated following the injection of cellular or protein antigens.

LITERATURE CITED

Baer, J. G., 1944, Immunité et reactions immunitaires chez les invertebrés. Schweiz. Zeits. fur Allg. Path. u. Bakt. 7: 442-462.

Bang, F. B., 1956, A bacterial disease of *Limulus polyphemus*. Bull. Johns Hopkins Hosp. 98: 325-338.

Butcher, E. O., 1930, The formation, regeneration and transplantation of eyes in Pecten *(Gibbus borealis)*. Biol. Bull. 59: 154-164.

Cameron, G. R., 1932, Inflammation in earthworms. Jour. Path. and Bact. 35: 933-972.

Campbell, D. H., 1957, Some speculations on the significance of formation and persistence of antigen fragments in tissues of immunized animals. Editorial in Blood 12: 589-592.

Cushing, J. E., 1957, Tissue transplantation in *Pecten irradians*. Abstract in Biol. Bull. 113: 327.

Cushing, J. E. and D. H. Campbell, 1957, Principles of Immunology. 344 pp. McGraw-Hill Book Company, New York.

Fisher, W. K., 1952, The Sipunculid worms of California and Baja California. Proc. U. S. Nat. Mus. 102: 371-450.

Glaser, O. C., 1910, The nematocysts of Eolids. Jour. Exp. Zool. 9: 117-142.

Hyman, L. H., 1955, The invertebrates: Echinodermata. 763 pp. McGraw-Hill Book Company, New York.

Jordan, H. E., 1938, Comparative hematology. Handbook of Hematology, Ed. Downey, H., Vol. I and II.

Kepner, W. A., 1943, The manipulation of the nematocysts of *Pennarig tiarella* by *Aeolis pilata*. Jour. Morphol. 73: 297-311.

Korschelt, E., 1931, Regeneration and transplantation. Berlin Gebruder Borntraeger, Vols. I and II.

Loeb, L., 1945, The biological basis of individuality. 711 pp. C. C. Thomas, Springfield, Ill.

Peebles, F. and D. L. Fox, 1933, The structure, functions, and general reactions of the marine Sipunculid worm *Dendrostoma zostericola*. Bull. Scripps Inst. Ocean., Tech. Series 3: 201-224.

Prizbram, H., 1926, Tierpfropfung, Die Transplantation der Korperabschnitte, Organe, and Keime. Die Wissenschaft 75: 304 p.p

Salt, G., 1957, Experimental studies in insect parasitism X. The reactions of some Endopterygote insects to an alien parasite. Proc. Roy. Soc. 147: 167-184.

Woods, K. R., E. C. Paulsen, R. L. Engle, Jr., and J. H. Pert, 1958, Starchgel electrophoresis of some invertebrate sera. Science 127: 519-520.

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RESETTING THE EUGLENA CLOCK WITH A SINGLE LIGHT STIMULUS*

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INTRODUCTION

As part of a comparative study of the formal properties of biological daily rhythms the writers wish to report here the details of some investigations on the rhythm of phototactic response in a single-celled microorganism, the flagellate *Euglena gracilis*. In previous papers the results of similar investigations on the eclosion rhythm in Drosophila (Burchard, 1958; Pittendrigh, 1958; Pittendrigh and Bruce, 1957 and 1958) and the activity rhythm in the hamster (Burchard, 1958; Pittendrigh and Bruce, 1958) are reported. Further comparative analyses are being pursued in this laboratory using cockroaches, lizards, birds and fungi. Workers in other laboratories (Hastings and Sweeney, 1958a, b; Ehret, 1958) have done experiments of a similar type using other microorganisms and have obtained similar results.

It has been observed—though perhaps not generally appreciated (c.f. Harker, 1958, page 50)—that for many endogenous daily rhythms the phase of a rhythm persisting in constant laboratory conditions may be shifted by a single light signal. The writers have previously noted (Pittendrigh and Bruce, 1958) several remarkable features of the way in which this phase-shifting occurs: (1) The new phase which is *ultimately* attained is uniquely determined by the timing of the "resetting" signal; that is, the disturbance does not act as a random perturbation. (2) The new steady-state condition is not attained immediately but only after several "transient" cycles. (3) The "period" of the transient cycles is characteristically longer than the period of the steady-state cycles if the resetting signal begins during the "day" phase of the cycle and shorter if the resetting signal begins during the middle or late night phase of the cycle. The former we shall hereafter call *delaying transients*; and the latter *advancing transients*.

The implications of the results of phase-shifting experiments have been discussed by the writers elsewhere (Pittendrigh and Bruce, 1957, 1958). We shall give here only the general philosophy underlying experiments of this type. Resetting light signals coming at any hour of the day or night are exogenous disturbances of a type not found in nature and hence have exerted no selective influence on the biological system controlling the assayed rhythm. Thus, any common characteristics in the formal properties of the response to resetting signals are suggestive of a common underlying mechanism.

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EXPERIMENTS

The nature of the endogenous rhythm in the phototactic response of Euglena, as well as the general techniques of culturing, measurement of the phototactic response, and recording have been previously described (Bruce and Pittendrigh, 1956). Reference should be made to that paper for additional details.

Cultures are grown in an organic medium (Hutner et al., 1950), washed, and resuspended in the inorganic medium previously described (Bruce and Pittendrigh, 1956). Minor modifications in the previously described test equipment have been made. Five similar test chambers have been constructed, allowing simultaneous recording from five cultures. Ten ml of culture are placed in a 50-mm-diameter Carrell flask for the experiments. These flasks are placed on a platform in a light-tight cabinet through which temperature-controlled air is circulated. Each test cabinet contains two lamps. One, located laterally to the culture, is a 7-watt incandescent "day lamp" (in place of the 4-watt fluorescents previously used), which is used to establish a light-dark (day-night) cycle and also as the resetting light in these experiments. The other lamp is a 6-volt microscope lamp located below the culture. A narrow vertical beam of light from the microscope lamp after passing through some heat-adsorbing glass intercepts a small part of the culture volume (about two per cent) and falls on a photocell. This lamp, called the test lamp, serves two functions. The light acts as the attracting stimulus which elicits the phototactic response and the transmitted light serves as a measure of the number of cells responding to the test light. The clock controlling the test lamp is a Flexopulse timer permitting variable test light cycles. In most of the experiments described here the test lights have been on for 20 minutes approximately every two hours, but not coming at exactly the same clock hours on successive days.

The output of the photocell is recorded on a chart which moves only when the test lights are on. The records illustrated in this paper are tracings of the actual records. Figure 1, for example, illustrates two days' record from a culture in so-called constant darkness (DD) in which the culture is exposed only to the test light every two hours. The first response on XII-10, which stays relatively constant (flat), indicates that the cells

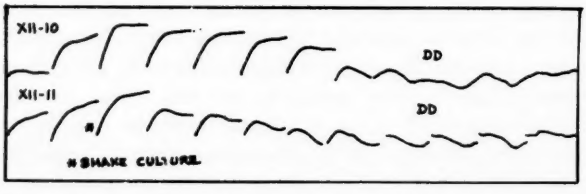

FIGURE 1. The figure shows the record of the phototactic responses of a culture to a 20-minute test light at approximately two-hour intervals for two days. In the experiment illustrated here the culture was removed from the test chamber momentarily at the time indicated by the asterisk, shaken up, and then replaced. There is little or no effect on the phototactic response or on the rhythm controlling the phototactic response. Compare with Figure 2.

are not responding to the light and is typical of a nighttime response. The second response—a rising line—corresponds to a decreasing output from the photocell (an increasing concentration of cells in the test-light beam) and represents a positive phototactic response typical of daytime responses. In all the other figures each day's record has been plotted twice, once to the right of, and once just below, the previous day's record so that the results of phase-shifting experiments may be more easily visualized.

The construction of five presumably identical test cabinets has revealed that the nature of the phototactic response depends in a very sensitive way on the precise details of the physical set-up. In contrast, however, the "clock" which controls the phototactic response is, like other biological clocks, remarkably stable and influenced only by certain particular exogenous factors. Consequently, although the *pattern* (the shape of the envelope of the phototactic responses) of phototactic responses in two aliquots

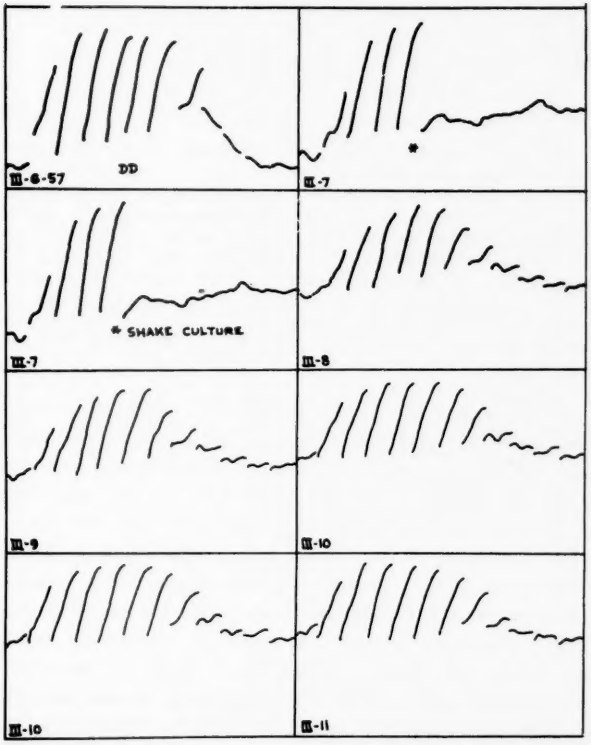

FIGURE 2. In this figure, as well as in all following figures, the record of the phototactic responses for each day is plotted twice, once to the right of, and once just below, the previous day's record. As in the experiment illustrated in Figure 1 the culture was shaken at the time indicated by the asterisk. Although there was a temporary effect on the nature of the following phototactic responses the phase of the rhythm was not affected. Compare with Figure 1.

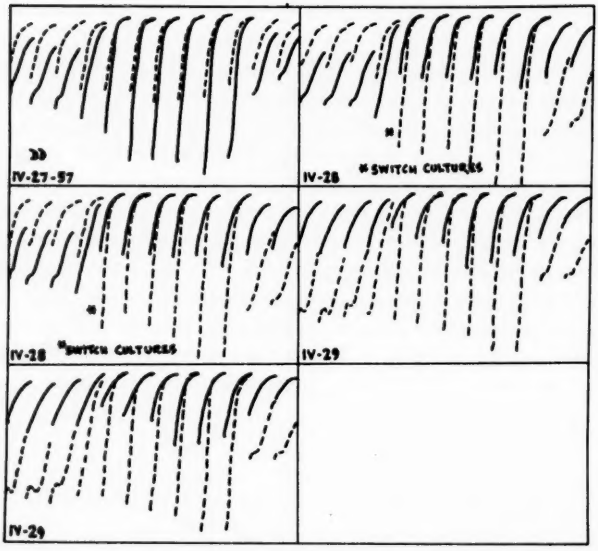

FIGURE 3. The record of the phototactic responses of two cultures—previously synchronized by a light-dark cycle—for three days in "constant darkness" (DD) is represented in the manner described in Figures 1 and 2. The difference between the *pattern* of the responses of the two cultures represented by the dashed and solid lines is primarily due to small differences in the physical (optical) characteristics of the two test chambers. This is evident from the results of switching the cultures between the two test chambers in the experiment represented here. The *pattern* of the phototactic responses (represented by the shape of individual response curves as well as by the shape of the "envelope" of the rhythm of responses) is characteristic of the test chamber, but the phase and period of the rhythm of responses are characteristic of the culture.

of the same culture may be quite different when tested in physical set-ups which differ only very slightly (figure 3), the rhythmicity—phase and period—of each culture is not influenced by switching the cultures between the two physical set-ups. Likewise, shaking a culture may (figure 2), or may not (figure 1) disturb temporarily the nature of the phototactic responses, but the phase of the rhythm is not affected in either case. The authors have previously suggested (Pittendrigh, 1958; Pittendrigh and Bruce, 1957; Bruce and Pittendrigh, 1957) that the periodic repetition of the test lights every two hours (or at some other interval) may entrain the endogenous rhythm to a 24-hour period via a frequency demultiplication effect. As a result of more extensive experiments it now seems clear that this effect does not ordinarily occur, at least to such a large submultiple (1/12th) of the driving frequency. Nevertheless, in spite of the relative stability of the endogenous rhythm to various exogenous factors it is, like other biological 24-hour rhythms, subject to control by light stimuli of a sufficient intensity and duration. This shows up very strikingly in the experiments now to be described.

Phase shifting with 12-hour light signals

Figure 4 illustrates the results of an experiment in which the phase of the phototactic rhythm has been shifted by means of a 12-hour light signal which starts at 24 + 10 hours after the last dawn of the previous LD (light-dark) cycle. The results of all experiments of this type are plotted with successive days underneath each other and with two successive days

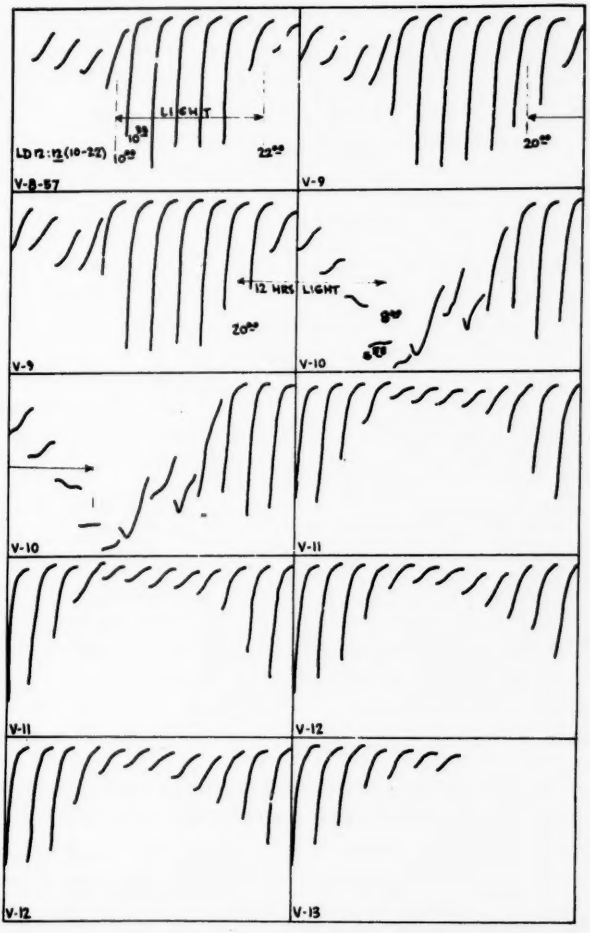

FIGURE 4. In this, as well as in the following figures, rhythms established by light cycles are reset (shifted in phase) by single light stimuli. In some cases the resetting light signal is given several days after the last dawn of the light signal. In the case illustrated here the culture receives a single 12-hour light stimulus beginning 24 + 10 hours after the last dawn of the previous light-dark cycle in which 12 hours of light from 10 to 22 were followed by *12* hours of dark from 22 to 10 − LD 12 : *12* (10–22).

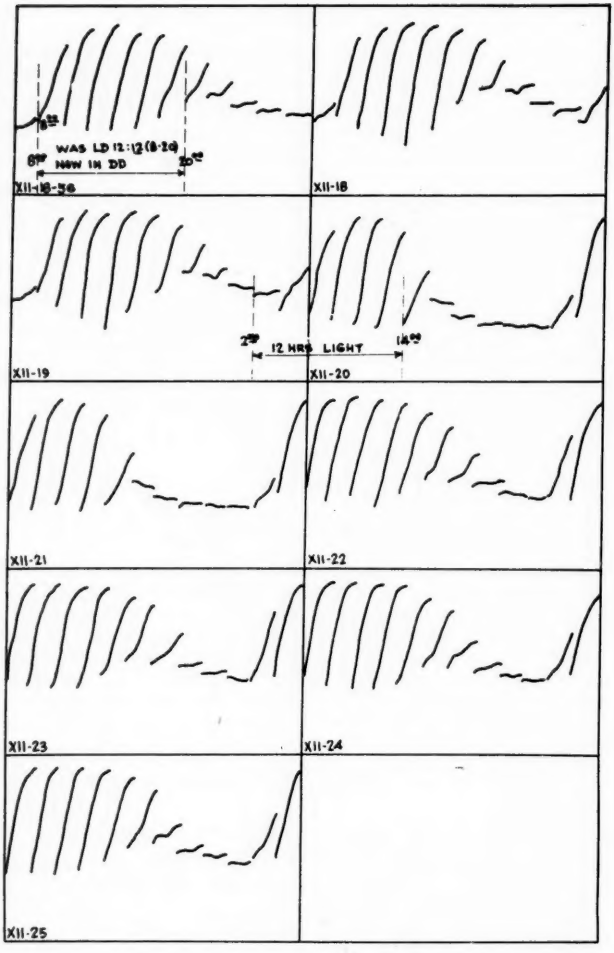

FIGURE 5. The first day shown is the steady state (DD) resulting from an LD 12:*12* (8–20) light cycle. The 12-hour resetting light signal begins at $3 \times 24 + 18$ hours after the last dawn.

plotted horizontally so that each day except the first and last is plotted twice. Each day shown runs from midnight to midnight, with the actual time of the beginning of the test lights shown on the figures. The first day shown in figure 4 represents the steady state resulting from an LD 12:*12* (10–22) cycle (12 hours of light from 10 to 22 and *12* hours of dark from 22 to 10). On the following day 12 hours of light are given starting 10 hours

"late"; subsequently the culture is returned to DD (the culture is exposed only to the test lights). Several points are worth noting:

1) The single light shock definitely shifts the phase of the endogenous rhythm.
2) A new steady state is not immediately attained; that is, the responses during the first, second, and third 24-hour periods after the start of the resetting light are all different, but the fourth 24-hour period is very much like the third.
3) The characteristic daytime responses occurring during the light period (day 1) and again in the dark during the day phase (day 2) are definitely delayed as a result of the resetting light and still further delayed two days after the resetting light. In this sense one can say that the 12-hour resetting light starting at hour 10 resets the phase of the rhythm with associated *delaying transients.*

Figure 5 illustrates the results of an experiment in which the phase of the rhythm is shifted forward with *advancing transients* as a result of a 12-hour light signal which begins at $3 \times 24 + 18$ hours after the last dawn of the previous LD cycle. The reader should especially note the existence of transients evidenced by the increasing amplitude of the phototactic response coming just after the start of the resetting light and at 24-hour intervals thereafter.

Other resetting experiments using 12-hour light signals have been done and can be summarized as follows:

1) Signals beginning within the first 10 hours after the last dawn or "extrapolated dawn" of the cycle will reset the phase of the rhythm by delays.
2) Signals beginning within the last 12 hours of the cycle will reset the phase of the rhythm by advances.
3) The existence of a transient approach to a new steady state is more clearly evident when the resetting signals begin during the middle of the cycle than when these signals begin either at the beginning or at the end of the cycle.

Phase shifting with 4-hour and 8-hour light signals

Experiments similar to those just described have been done using light signals of 4 hours duration rather than 12 hours. Figures 6 and 7 represent the results of two such experiments in which the beginning of the 4-hour light signal comes at hours 6 (figure 6) and 10 (figure 7). The 4 hours of light beginning at hour 6 fall entirely within the day phase of the cycle and there is no evidence that the phase of the rhythm has been shifted at all. On the other hand, 4 hours of light beginning at hour 10 does shift the phase of the rhythm several hours. The experiments illustrated, together with similar ones in which the resetting signal begins at hours 3, 15, 18, and 21 can be summarized by the statement that a 4-hour light

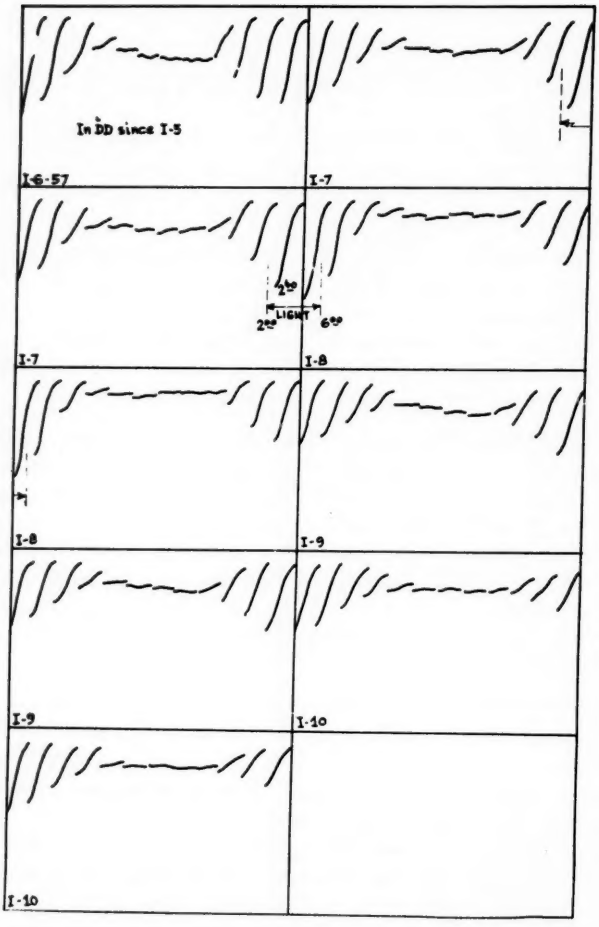

FIGURE 6. The first day shown is the steady state (DD) resulting from an LD 12:12 (20-8) light cycle. The 4-hour resetting light signal begins 3 times 24 + 6 hours after the last dawn.

signal falling entirely within the day phase of the cycle apparently does not shift the phase of the rhythm, whereas if part of the resetting light falls within the night phase of the cycle the phase of the rhythm is shifted.

If 8 hours of resetting light is used to shift the phase of the rhythm, analagous results are obtained. Figure 8 shows the results of an experiment using 8 hours of light starting at hour 4 of the cycle and hence falling entirely within the day phase of the cycle. We cannot conclude that the phase of the rhythm has been shifted, although 8-hour resetting signals starting at hours 9 or 12 (see figure 9) *will* shift the phase of the rhythm.

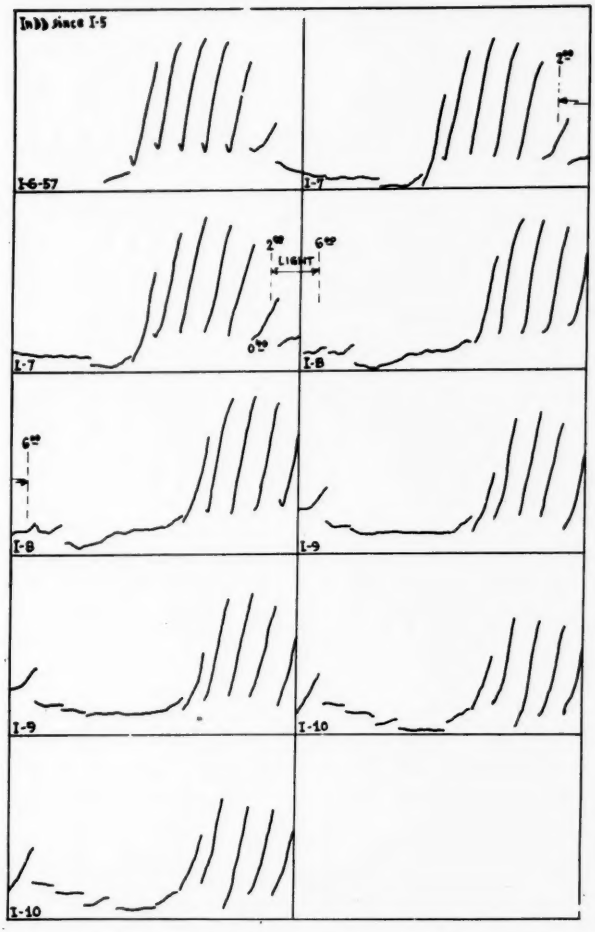

FIGURE 7. The first day shown is the steady state (DD) resulting from an LD 12:*12* (16–4) light cycle. The 4-hour resetting light signal begins 3 times 24 + 10 hours after the last dawn.

It is clear from figures 7 and 9 that a single 4-hour or 8-hour light signal may shift the phase of the rhythm, though not as efficiently as a 12-hour signal.

DISCUSSION

The significance of the experiments described here is primarily the following. They show that certain formal non-adaptive features of the response (phase shifting) of an endogenous rhythm to single perturbations with light—previously observed in higher organisms (Drosophila, ham-

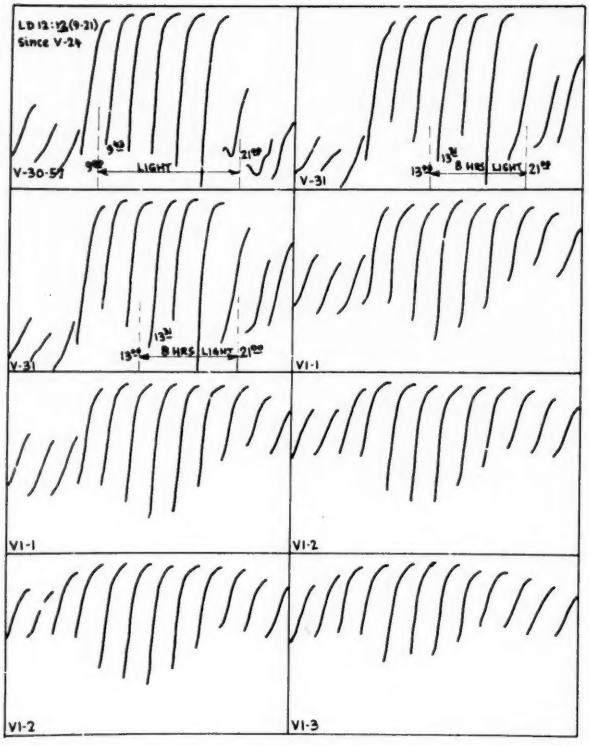

FIGURE 8. The first day shown was the last of several in LD 12:*12* (9-21). The 8-hour resetting light signal begins 24 + 4 hours after the last dawn.

ster)—are also found in the single-celled organism Euglena. Euglena is not an ideal organism with which to make further detailed analysis of the phase-shifting response to various types of light perturbations for the following reasons:

1) The rhythm (phototactic response) can only be assayed by short repeated exposures to light.
2) The precision with which phase shifts can be measured depends on the repetition frequency of the test-light signal, which cannot conveniently be made less than about 2 hours.
3) The assayed periodicity consists of an alternation between characteristically "daytime" responses and "nighttime" responses, with no well-defined unique point in the cycle such as is characteristic of the eclosion rhythm in Drosophila or the onset of running activity in rodents. The quantitative characterization of transients in Euglena is therefore more difficult to define satisfactorily.

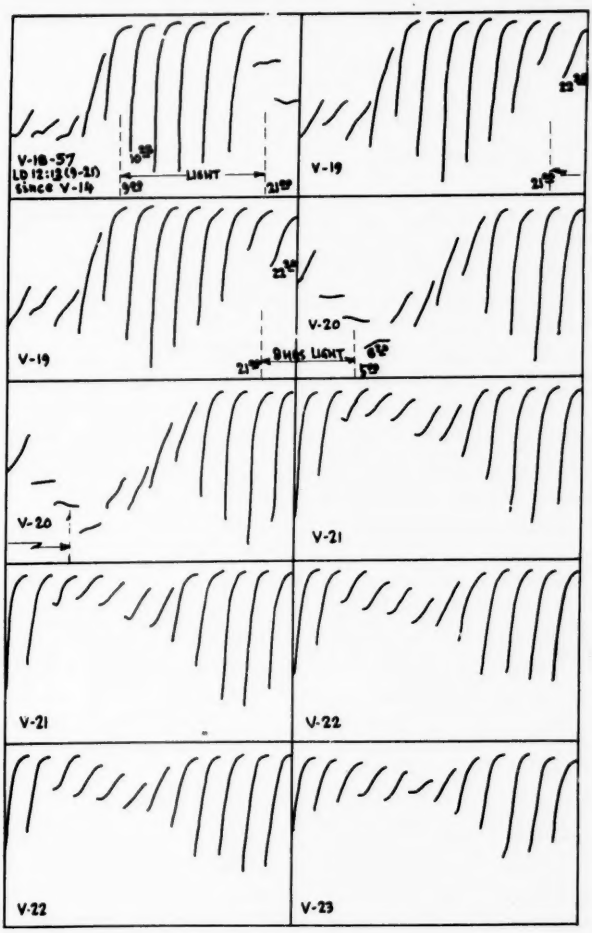

FIGURE 9. The first day shown was the last of several in LD 12:*12* (9–21). The 8-hour resetting light signal begins 24 + 12 hours after the last dawn.

It is for this reason that we do not wish to make a more detailed formal comparison of "Resetting in Euglena" with "Resetting in the fly or the hamster" but rather to emphasize the striking qualitative facts that there does exist a *transient approach to new phase* and that this may occur either with *delaying* transients or *advancing* transients when the phase of the Euglena rhythm is shifted with a single light stimulus. These formal features of the "clock system," which are explained neither from adaptive considerations, nor from vague requirements of simplicity, are the principal argument for the hypothesis of a common clock mechanism in diverse organisms.

LITERATURE CITED

Bruce, V. G. and C. S. Pittendrigh, 1956, Temperature independence in a unicellular "clock." Proc. Nat. Acad. Sci. 42: 676–682.

1957, Endogenous rhythms in insects and microorganisms. Amer. Nat. 91:179–195.

Burchard, J. E., 1958, Resetting a biological clock. Ph.D. Thesis, Princeton University.

Ehret, C. F., 1958, The induction of phase shift in cellular rhythmicity by far ultraviolet and its restoration by visible radiant energy. *In* Photoperiodism and Related Phenomena in Plants and Animals. (R. B. and A. R. Withrow, ed.) Am. Assoc. Adv. Sci. (in press, 1958).

Harker, J., 1958, Diurnal rhythms in the animal kingdom. Biol. Rev. 33: 1–52.

Hastings, J. W. and B. M. Sweeney, 1958a, A persistent rhythm of luminescence in *Gonyaulax polyedra*. (In press)

1958b, The Gonyaulax clock. *In* Photoperiodism and Related Phenomena in Plants and Animals. (R. B. and A. R. Withrow, ed.) Am. Assoc. Adv. Sci. (in press, 1958).

Hutner, S. H., L. Provasoli, A. Schatz, and C. P. Haskins, 1950, Some approaches to the study of the role of metals in the metabolism of microorganisms. Proc. Amer. Phil. Soc. 94: 152–168.

Pittendrigh, C. S., 1958, Perspectives in the study of biological clocks. *In* Symposium on Perspectives in Marine Biology. University of California Press, Berkeley. (In press)

Pittendrigh, C. S. and V. G. Bruce, 1957, An oscillator model for biological clocks. *In* Rhythmic and Synthetic Processes in Growth. pp. 75–109. Princeton University Press.

1958, Daily rhythms as coupled oscillator systems; and their relation to thermo- and photoperiodism. *In* Photoperiodism and Related Phenomena in Plants and Animals. (R. B. and A. R. Withrow, ed.) Am. Assoc. Adv. Sci. (in press, 1958).

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SERUM ELECTROLYTE LEVELS IN HIBERNATING MAMMALS*

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INTRODUCTION

Reports on serum electrolyte changes with hibernation have been consistent in indicating an elevation of serum magnesium (Suomalainen, 1939; McBirnie, et al., 1953; Riedesel, 1957), but inconsistent with respect to other electrolytes. For example: hypo- and hyper-calcemia have been described with hibernation in the European marmot (Adler, 1926; Ferdmann and Feinschmidt, 1932) but no change in the serum calcium with hibernation of the hedgehog (Suomalainen, 1939); increased serum potassium with hibernation of the American woodchuck (McBirnie, et al., 1953), but decreased serum potassium (Suomalainen, 1953), or no change (Biorch, et al., 1956) with hibernation of the hedgehog. These contradictions may result from species characteristics or differences in the depth of hibernation. Accordingly, the present study was designed to describe species differences in serum electrolytes as related to depth or type of hibernation. An earlier report by the senior author describes elevation of serum magnesium with the lowering of body temperature in the little brown bat (*Myotis lucifugus*) (Riedesel, 1957). This presentation records serum calcium and potassium measurements on four species of hibernating animals, including two species of bats, hamsters, and thirteen-lined ground squirrels.

PROCEDURE AND TECHNIQUES

Simultaneous serum calcium, potassium, specific gravity and blood hematocrit determinations were made on animals bled by decapitation. In the case of the bats, serum electrolyte and specific gravity determinations were made on samples obtained by pooling the blood of five animals. Potassium determinations were made by flame photometry. The spectrophotometric method of Natelson and Penniall (1955) was employed for the analysis of total serum calcium. Hematocrit values on the small bats were determined by the use of Van Allen hematocrit tubes. The Wintrobe method was employed for hematocrit determinations on the other animals. In either case the blood was centrifuged at 2800 r.p.m. for 45 minutes. The serum specific gravities were determined by the method of Van Slyke, et al. (1950). Esophageal temperatures of the small bats were measured on a Rubicon potentiometer with a copper-constantan thermocouple just before they were sacrificed. When initial stages of hibernation were studied, esophageal

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temperatures were measured at 30 minute intervals. The process of cooling to 18 and 13°C. required from 30 minutes to 10 hours. During awakening from hibernation continuous measurements of esophageal temperatures were made. The warming to 13 and 18°C. required from 5 to 30 minutes depending upon the initial depth of hibernation. The series were limited to males in the case of the rodents, but contained male and female with both species of bats. With them serum electrolyte and hematological data were analyzed separately, but sex differences were not found (p values were all greater than 10 per cent). Therefore, the electrolyte and hematological data for bats are combined data from males and females. Each value presented in tables 1 and 2 represents the mean of at least four samples (20 animals).

TABLE 1

SERUM ELECTROLYTE AND HEMATOLOGICAL DATA OF *MYOTIS LUCIFUGUS* IN COLD EXPOSURE

	Calcium mEq/l	Potassium mEq/l	Specific gravity	Hematocrit
Active				
4 days				
warm room (24°C)	4.46	6.37	1.0226	46.0
	(0.28)*	(0.93)	(0.0008)	(0.6)
18–32 hrs				
cold room (6°C)	3.40	7.24	1.0229	
	(0.31)	(0.60)	(0.0006)	
Hibernating				
12 hrs		7.05	1.0228	50.1
		(0.31)	(0.0004)	(1.6)
18 hrs	3.28	6.81	1.0222	46.7
	(0.41)	(0.05)	(0.0003)	(2.6)
32 hrs	4.00	6.55	1.0223	
	(0.92)	(0.60)	(0.0003)	
2 days	3.79	6.52	1.0239	48.8
	(0.54)	(0.55)	(0.0014)	(4.1)
4 days	4.28		1.0243	48.3
	(0.36)		(0.0003)	(4.1)
10 days	2.40	6.89	1.0247	
	(0.42)	(0.60)	(0.0009)	
9 wks	3.12	7.10	1.0294	51.8
	(0.40)	(0.44)	(0.0002)	(0.9)

*Standard deviation.

The source and maintenance of these specimens were described earlier (Riedesel, 1957). Triplicate determinations were made on all samples obtained from rodents. Small sample statistical methods were used to analyze the data and a five per cent or less level of confidence was accepted as a significant difference.

RESULTS

The data describe primarily homeostasis of serum calcium and potassium levels during hibernation, with some exceptions. Two control groups and

TABLE 2

SERUM POTASSIUM AND HEMATOLOGICAL DATA OF *MYOTIS LUCIFUGUS* IN STAGES OF HIBERNATION

Conditions	Potassium mEq/l	Specific gravity	Hematocrit
Active, 4 days	6.37	1.0226	46.0
	(0.94)*	(0.0008)	(0.6)
Cooled to E.T. of 17–20°C	7.20		49.0
	(0.36)		(2.8)
Cooled to E.T. of 11–13°C	7.25	1.0273	48.2
	(0.18)	(0.0006)	(2.8)
Warmed to 13°C	7.15	1.0281	50.0
	(0.55)	(0.0007)	(4.7)
Warmed to 18°C	7.07	1.0283	55.2
	(0.42)	(0.0006)	(2.6)
Active, 1 hour	7.80	1.0284	52.5
	(0.29)	(0.0001)	(0.9)

*Standard deviation.
E.T.— Body temperature measured in the esophagus.

twelve groups of little brown bats exposed to the cold were studied. In all cases mean calcium values for cold-exposed animals, hibernating and non-hibernating, were lower than control values (figure 1 and table 1). The differences between the mean values of the group active four days and the groups which hibernated ten days and nine weeks respectively are significant at the 99 per cent level of confidence (table 1). The serum potassium values did not change significantly; however, the mean values during hibernation were consistently higher (tables 1 and 2). The cell/plasma ratio and serum specific gravity usually did not change consistently (tables 1 and 2).

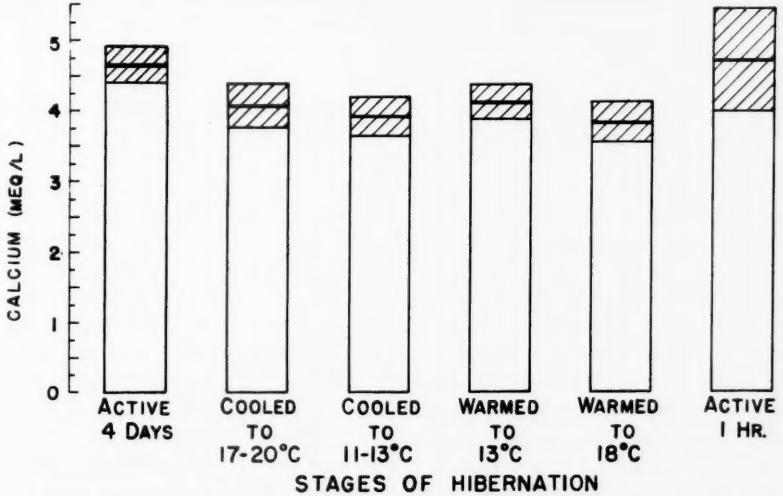

FIGURE 1. Serum calcium measurements of *Myotis lucifugus* in stages of hibernation. (Shaded area represents one standard deviation above and below the mean.)

The results for the first hour after arousal (table 2) showed unexpected trends. It is known that the first hour after arousal is characterized by drastic changes in homeostatic mechanisms. During this period the vascular bed is opened, the wings are spread, the spleen contracts, the state of "emergency" results in hypercoagulability of the blood, rapid changes in serum magnesium occur, and the animal probably becomes more dehydrated. The high values of potassium, specific gravity and hematocrit are consistent with these changes (table 2, active one hour).

TABLE 3

SERUM ELECTROLYTE AND HEMATOLOGICAL DATA OF HIBERNATORS IN COLD EXPOSURE

		Active in warm (24°C)		Active in cold (6°C)		Hibernating 2 days	
		Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.
Big Brown Bat n = 4 to 30	Potassium (mEq/1)	6.16	0.19			5.88	0.18
	Specific gravity	1.0206	0.0005			1.0240	0.0004
	Hematocrit	53.2	2.0			44.7	2.7
Ground Squirrel n = 4 to 8	Calcium (mEq/1)	4.66	0.30	4.89	0.96	4.97	0.30
	Potassium (mEq/1)	5.77	0.28	8.16	1.1	6.5	0.54
	Specific gravity	1.0235	0.0002	1.0245	0.0019	1.0232	0.0007
	Hematocrit	46.8	2.7	51.0	6.9	48.4	2.1
Hamster n = 2 to 4	Calcium (mEq/1)	5.35	0.02	5.78	0.18	5.59	0.10
	Potassium (mEq/1)	8.63	0.05	6.52	0.26	9.46	0.0*
	Specific gravity	1.0245	0.0010	1.0232	0.0071	1.0234	0.0045
	Hematocrit	34.9	21.2	55.9	4.0	57.4	3.4

*n = 2.

In contrast to the little brown bat, data on the big brown bat (*Eptesicus fuscus*) did not demonstrate consistent increase in the serum potassium (table 3). In the case of the ground squirrels, cold exposure and hibernation did not alter the serum calcium, specific gravity or blood hematocrit. Control and hibernating ground squirrels also had similar serum potassium values. A 40 per cent increase in the mean potassium was observed with animals not hibernating during exposure to the cold. The two hamsters exposed to the cold had serum potassium levels much lower than their warm room controls, but a larger sampling of this species is necessary to define the significance of this response. The increases in the serum potassium and specific gravity with hibernation are similar to those observed with the little brown bat and ground squirrel. No changes were observed in the serum calcium with hibernation of the hamsters.

DISCUSSION

The ranges of the serum electrolyte concentrations of the control animals in this study are similar to those reported for mammals which cannot hibernate (Albritton, 1952). However, decreases in calcium and apparent increases in potassium concentrations were observed with hibernation. It is of particular interest to note the similarities between the responses of the body temperature of the hibernators and the changes in these serum electrolyte concentrations. The little brown bat provided the first consistent evidence of low serum calcium with hibernation. The data in figure 1 suggest that the serum levels observed during hibernation may vary with depth of hibernation.

Observed similarities and differences in serum potassium levels may be related to differences in body temperature during cold exposure. Earlier studies have described lowered body temperatures for active bats and ground squirrels exposed to the cold whereas active hamsters do not exhibit such lowering of body temperatures (Folk, 1957). Regarding changes in serum potassium, active hamsters were once again different in that the data indicate a decrease in the serum potassium concentration with cold exposure. The active bats and ground squirrels had an increase under the same conditions. During hibernation all three species are consistent in having higher serum potassium.

The cause and effect relationship of the electrolyte changes observed during cold exposure and hibernation cannot be described completely until further studies have been made with emphasis on such pertinent factors as: renal excretion of cations, effect of cooling and pH changes on the ion binding power of intracellular proteins, adequacy of circulation during various phases of hibernation, activity of the adrenal cortex, and activity of cell membrane electrolyte transfer systems.

SUMMARY

Earlier work has shown an increase in serum magnesium to be a characteristic of hibernation with the magnitude of the change by species in this order: ground squirrel > bat > hamster. The present study describes another prominent change in serum electrolytes: namely, a drop in serum calcium with active and hibernating little brown bats exposed to the cold. Active bats and ground squirrels both appear to have a rise in serum potassium during cold exposure with less definite rise in hibernation. The hamster, as usual, shows a different response, in this case a decrease in serum potassium when active during cold exposure. Consistent with the other species, the hamster shows a slight rise in this ion in hibernation. On the whole, except for serum calcium in the little brown bat, and serum magnesium changes, the present experiments support the view that hibernation is characterized by the presence of serum electrolytes at control levels or slightly higher.

LITERATURE CITED

Adler, Leo, 1926, Der Winterschlaf. Handbuch Normal. u. Pathol. Physiol. 17: 105–133.

Biœrch, G., B. Johansson and S. Veige, 1956, Some laboratory data on hedgehogs, hibernating and non-hibernating. Acta Physiol. Scand., 37, Fasc. 4: 281–294.

Ferdmann, D. and O. Feinschmidt, 1932, Der Winterschlaf. Ergebn. Biol. 8: 1–75.

Folk, G. E., Jr., 1957, Twenty-four hour rhythms of mammals in a cold environment. Amer. Nat. 91: 153–166.

Lyman, Charles P. and Paul O. Chatfield, 1955, Physiology of hibernation in mammals. Physiol. Rev. 35: 403–425.

McBirnie, J. E., F. G. Pearson, G. A. Trusler, H. H. Karachi and W. G. Bigelow, 1953, Physiological studies of the groundhog (*Marmota monax*). Can. J. Med. Sci. 31: 421–430.

Natelson, S. and R. Penniall, 1955, Colorimetric estimation of ultramicro quantities of calcium in human serum as the complex with alizarin. Anal. Chem. 27: 434–437.

Riedesel, Marvin L., 1957, Serum magnesium levels in mammalian hibernation. Trans. Kans. Acad. Sci. 60: 99–141.

Suomalainen, Paava, 1939, Hibernation of the hedgehog, VI. Serum Mg and Ca. Artificial hibernation also a contribution to chemical physiology of diurnal sleep. Annal. Acad. Sci. Fennicae. Ser. A. Tom. 53, No. 7, 1–71.

1953, Proc. Finnish Acad. Sci & Letters: 131.

Van Slyke, Donald D., P. B. Hamilton, V. P. Dole, K. Emerson, Jr. and R. M. Archibald, 1950, Measurement of specific gravities of whole blood and plasma by standard copper sulfate solution. J. Biol. Chem. 183: 305–330.

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LETTERS TO THE EDITORS

Correspondents alone are responsible for statements and opinions expressed. Letters are dated when received in the editorial office.

SELECTION FOR SUBTHRESHOLD DIFFERENCES AND THE ORIGIN OF PSEUDOEXOGENOUS ADAPTATIONS

The callosities on the sole of the human foot have long stood as examples for a whole group of phenomena whose evolutionary origin seemed difficult to understand if one holds neo-Darwinian views. These callosities do not appear first when the foot is exposed to the external stress of walking but are present already in the embryo. Phylogenetically, it is supposed that when the primates ancestral to Man began to walk on their hind extremities their soles developed callosities at those areas most exposed to friction. Later, what was at first only an exogenous adaptation, to use Waddington's (1958) term, was automatically produced by the evolved genotype so that the callosities are now pseudoexogenous.

In recent years, two different though not mutually exclusive hypotheses have been adduced to explain the origin of pseudoexogenous adaptations. One which has been given the name "Baldwin effect" assumes in the case of the human foot sole that in addition to the somatic acquisition of callosities, mutations for their autonomous development happened to occur and that they were selected under cover of the exogenously induced phenotypes. This explanation obviously does not make it clear why selection should have favored a genetically anchored system of callosities when their individual acquisition was assured by the long existent somatic reactivity. The other hypothesis of the origin of pseudoexogenous adaptations also starts with organisms who respond somatically by individual adaptation to the evolutionary new stresses on the foot. It then invokes selection of genotypes which insure a greater degree of constancy than at first, of the adaptation from individual to individual. This stabilization of the genetic constitution toward uniformly strong expression of the adaptability to the specified environment is believed to bring the organism automatically into a position in which development even in the absence of the specified environment produces the trait in question. Waddington has called this transformation from individual response to spontaneous production "genetic assimilation."

He has carried out experiments in which such transformations seemed to have been observed directly (for the latest discussion and earlier references see Waddington, 1958). By exposing larvae of *Drosophila melanogaster* to heat shock, or eggs to ether, he produced in some of them the development of phenocopies for special venation types or the bithorax condition. Selecting for those individuals which had responded by phenocopy

production to the special environmental stimulus, he was able in successive generations to increase the frequency of the response. On inspection of branch populations of the selected lines for the presence of vein or bithorax-traits in the absence of the heat or ether environment, he observed that the traits were now produced spontaneously in some individuals. This spontaneous production was gene-controlled.

As stated above, Waddington's interpretation of his results in terms of "genetic assimilation" involves three separate processes: (1) the initial reactivity of the developing organism to respond to a new environment, B, by morphological changes, (2) a selection of genotypes which assures the constancy and strong expressivity of the response in environment B, and (3) the crucial automatic transformation of the mechanism for (2) into one which produces the morphological changes even in the absence of environment B. The phenomena (1) and (2) do not offer particular difficulties but process (3) is not an obvious one. Its existence has been deduced by Waddington from his picture of the epigenetic landscape with its canalized routes of development. This picture has had the value of suggesting the phenocopy-selection experiments in Drosophila but as in the case of other models its applicability is not evidence for its validity.

It seems to me that a *single* cause may underly the origin of pseudo-exogenous adaptations. If, in a population, genes are present which produce a certain phenotype in *both* environments A and B, and if selection for these genotypes can be accomplished initially in environment B only, then, by definition, selection in B will accomplish production of the trait in both A and B. The possibility of existence of the postulated genotypes is a matter of common genetic experience. So is the existence of genotypic differences which remain phenotypically indistinguishable in one environment but can be discerned in another. We may speak of them as subthreshold differences in A and supra-threshold differences in B.

Two examples for the mechanism of selection for subthreshold differences will now be given.

Subthreshold differences at a single locus

Assume a population of *Drosophila melanogaster* in which the mutant allele *ci* (cubitus interruptus, a venation type) occurs with frequency $q = 0.001$ and the wildtype isoallele $+^C$ with the frequency $p = 0.999$ (Stern and Schaeffer, 1943). Under panmixis *ci/ci* homozygotes will occur in the proportion 1:1,000,000 only so that in medium sized populations of the order of ten thousand individuals, they will most likely be absent. Heterozygous $+^C/ci$ flies will occur at a rate of 1998 per million and will therefore be present in medium sized populations. Heterozygotes developing at a temperature A of 26°C are indistinguishable from homozygous wildtype flies $+^C/+^C$ so that the population though consisting of $+^C/+^C$ and $+^C/ci$ individuals, is phenotypically uniform. At a temperature B of 14°C, the $+^C/+^C$ homozygotes continue to develop into wildtype flies but more than ten per cent of the $+^C/ci$ heterozygotes produce the mutant phenotype. Ob-

viously, phenotypic selection which at 26° is not possible, becomes feasible at 14°C. Selection and interbreeding of individuals showing the mutant phenotype will result in segregation of *ci/ci* homozygotes. These produce the mutant phenotype at both temperatures A and B.

Subthreshold differences in a polygenic system

As a model, Wright's (1934) polydactylous guinea pig genotypes may be used. Slightly schematizing, we may postulate a system of four pairs of additively acting genes responsible for the phenotypic difference between the normal three toed and the polydactylous four toes phenotypes. If A′, B′, C′ and D′ are alleles which do not favor development of polydactyly, and A^2, B^2, C^2 and D^2 are alleles which do so, the breeding data can be interpreted in terms of a threshold level. In a standard environment, A, presence in the genotype of at least 6 genes with the superscript 2 leads to polydactyly while presence of from 0 to 5 such genes uniformly causes the development of three toes.

Environmental circumstances are known which shift the threshold for the effectiveness of the polydactyly-favoring alleles. Be it assumed that in environment B polydactyly is produced by a minimum of 4 alleles with the superscript 2. Consider a population in which the frequencies q of each of the A^2, B^2, C^2 and D^2 are 0.06 and those of A′, B′, C′ and D′ are p = 0.94. Then the proportion of genotypes with 6 or more alleles of type 2 is approximately 1 : 1,000,000 so that in medium sized populations living in environment A, polydactylous individuals will most likely be absent. Individuals possessing 4 and 5 alleles with action favoring polydactyly occur at a rate of over 746 per million and will therefore, be present in medium sized populations. In environment A these individuals are indistinguishable from three-toed guinea pigs with less than 4 polydactyly favoring alleles so that the population is phenotypically, uniformly non-polydactylous. In environment B, the embryos with less than 4 of the alleles continue to develop into three-toed animals but those with 4 or more of the alleles become polydactylous. Selection and interbreeding of individuals, showing the polydactylous phenotype (e.g., $A'A^2B^2B^2C'C^2D'D^2 \times A'A^2B'B^2C'C^2D'D^2$) will result in segregation of zygotes with 6 or more polydactyly favoring alleles, e.g., $A'A^2B^2B^2C'C^2D^2D^2$, $A^2A^2B^2B^2C^2C^2D'D^2$, etc. These produce polydactyly in both environments A and B.

DISCUSSION

The two models which involve no phenotypic differences in one environment (for either two genotypes at a single locus or for a variety of genotypes depending on several loci), show how selection can work in another environment if it permits discrimination of the different genotypes. In nature, single locus situations involving probably multiple alleles and polygenic situations with multiple alleles at more than one locus should be available to provide the material for selection in environments which lower the threshold of phenotypic discrimination. Goldschmidt and Piternick

(1956, 1958) and Landauer (1957), among others, have shown how the genotype determines the reaction to produce specific phenocopies and the former authors particularly have stressed the existence of genetic differences within populations, which remain below the threshold of effectiveness in one environment and rise above it in others. Waddington, himself, in his discussion of a certain experiment of Bateman (1956, quoted by Waddington, 1958) has clearly recognized the significance of subthreshold genotypes in "genetic assimilation." Bateman had found a low percentage of spontaneous crossveinless individuals in her initial population and by selection had built up a crossveinless stock which appeared identical in genotype to an "ssismilated" stock derived after temperature treatment. Waddington writes: "What the environmental treatment has done *in this case* is to reveal subthreshold concentrations of those genes, and thus made it possible for selection to get a hold" (1958, page 178, italics mine). The present suggestion merely places this explanation beyond the special case on a general level.

In summary, the main thesis of the proposed explanation for the origin of pseudoexogenous adaptations is that these traits are not first somatically acquired on the basis of the *general* genotype of the species and then stabilized and "assimilated" by selection for specific genotypes. Rather, these specific genotypes are below the threshold of effectiveness in the ancestral environment but above it in the evolutionary new one. They now acquire a positive selective value. Interbreeding of bearers of these genotypes produces by the conventional mechanisms of Mendelian combination homozygotes or other "higher" genotypes whose effectiveness is above the threshold of effectiveness in both the ancestral and the new environment.

LITERATURE CITED

Goldschmidt, R. and L. K. Piternik, 1956, New experiments on chemical phenocopies. Proc. Nat. Acad. Sci. 42: 299–304.

1957, The genetic background of chemically induced phenocopies in Drosophila. I. J. Exp. Zool. 135: 127–202; II. J. Exp. Zool. 136: 201–228.

Landauer, W., 1957, Phenocopies and genotype, with special reference to sporadically-occurring developmental variants. With a discussion by R. Goldschmidt. Amer. Nat. 91: 79–94.

Stern, C. and E. W. Schaeffer, 1943, On wild-type iso-alleles in *Drosophila melanogaster*. Proc. Nat. Acad. Sci. 29: 361–367.

Waddington, C. H., 1958, The Strategy of the Genes. ix + 262 pp. The Macmillan Co., New York.

Wright, S., 1934, The results of crosses between inbred strains of guinea pigs, differing in number of digits. Genetics 19: 537–551.

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ANOPHTHALMIC ALBINO: A NEW MUTATION IN THE SYRIAN HAMPSTER, *CRICETUS (MESOCRICETUS) AURATUS*

The usual albino hamster is of the type recently elucidated by Robinson (1957), and designated by him as c^d. It is inherited as a recessive. These animals are wholly white at birth. The eyes open at about 10–12 days post partum, are functionally normal, and exhibit the typical albino pink coloration. At about three weeks of age, however, a faint gray pigmentation begins to invade the skin of the ears, and this steadily deepens until, in adults, it is as dark as that observed in the ears of normal animals. Later in life pigment often appears in the skin of the genital areas being particularly visible in some cases on the prepuce or the scrotum of males. The coat remains white.

In anophthalmic albinos, the eyelids open at about 12-14 days post partum, but examination reveals only raw tissue beneath them. In most of the cases observed, they soon close again, and a yellowish exudate forms at their corners. They seldom open in later life. The coat is white. The

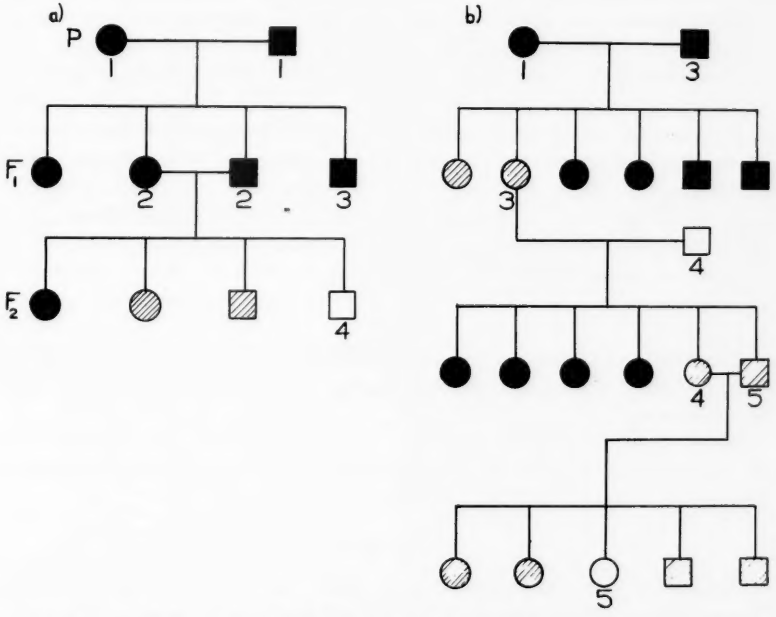

FIGURE 1. Two pedigrees of anophthalmic albino: a) the first appearance of the mutation; b) extraction of anophthalmic albino from c^d heterozygotes, showing the non-allelic character of these genes. Code: circles are females, squares males. Solid figures are normal phenotype, lined are c^d phenotype, blank are anophthalmic albinos. Small *an* has been used to represent anophthalmia. Probable genotypes of some of the animals directly involved are, for females: ♀2, $+//c^d+//an$; ♀3, $c^d//c^d+//+$; ♀4, $c^d//c^d+//an$; ♀5, $c^d//c^d$ *an*$//an$; for males: ♂2, $+//c^d+//an$; ♂3, $+//c^d+//+$; ♂4, $+//c^d$ *an*$//an$; ♂5, $c^d//c^d+//an$.

skin of the ears, unlike that of Robinson's albino, is a translucent pink, plainly showing the blood vessels. No pigment appears in the genital areas. Albinism is total, and remains so throughout life.

In the spring of 1956, the authors bred a pair of hamsters, from unrelated stocks. The male came of a known strain, in which no albinism had been recorded; the female's antecedents are unknown.

No albinism appeared in F_1. However an F_2 litter of eight, born August 27, 1956, included three albinos: two of the c^d phenotype described above, the third, a male, the first anophthalmic albino.

Beginning at the age of about ten weeks, the anophthalmic male was bred several times without result. His sire and dam were then remated, giving seven young of which five were albinos. Two of three surviving albinos were anophthalmic.

A normal female, sibling of the anophthalmic male, was then bred to her sire. Six of the nine young produced were albinos, of which four survivors were all anophthalmic.

Breeding experiments indicate that anophthalmic albino is recessive to normal; and, since normal coat color appears in the offspring of c^d females by anophthalmic males, that it is due to a genetic factor which is not an allele of c^d. Eleven anophthalmic albinos have been observed to date. In no case has either anophthalmia or total albinism appeared separately.

In the light of these facts, it seems possible that anophthalmic albinism may be caused by a gene for anophthalmia with pleiotropic effects resulting in the complete suppression, in the homozygote, of the effects of the normal color genes.

Breeding experiments are being continued, and histological investigation is also in progress. The latter is planned to include an examination of the ontogeny of this condition in embryos.

SUMMARY

Anophthalmic albino, an apparently new mutation in the Syrian Hamster, first appeared in 1956. The coat is white, the skin wholly unpigmented. Eyes are absent.

Breeding experiments indicate that this condition is recessive, and that it is not an allele of c^d. Further experiments, including histological investigation of this mutation, are in progress.

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LITERATURE CITED

Robinson, R., 1957, Partial albinism in the Syrian Hamster. Nature 180: 443–444.

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X-RAY DIFFRACTION OF OTOLITHS AND EGG SHELLS OF BIRD AND REPTILE

Many studies have been made of the crystal form of calcium carbonate in egg shells of birds (Romanoff and Romanoff). The egg shell crystals of four species of testudinate turtles have been studied (Young). Observations of the crystals in otoliths of many vertebrates have been published including a recent paper containing a review of several previous reports (Carlstrom and Engstrom). Studies on reptilian otoliths, however, have been notable exceptions. Only in the lizard have the otoliths been reported to be of crystalline structure, but with no clear description as to the kind or form of crystal (Tenaglia). Crystals of calcium carbonate occur in different polymorphic forms two of which are found in animal tissues, being either calcite or aragonite. Egg shells and otoliths of birds studied have been found to be of calcite. The egg shells of reptiles (turtles) are reported as being of aragonite.

In our laboratories, we had the opportunity to examine the egg shells and otoliths of the same domestic white leghorn hen and the same turtle, *Emys blandingi*. The inner shell membranes were removed, the shell air dried, then finely powdered. Otoliths were dissected out, cleaned of other tissue, then air dried and finely powdered. No fixatives were used. The powdered material was mounted in an x-ray diffraction apparatus and photographs made. Comparison of the films with those of known diffraction patterns of the polymorphic forms revealed the shell and otolith of the hen to be composed of calcite as was expected. The shell and otolith of the turtle were of the aragonite form. This, too, was expected as the egg shell of one of the species studied by Young was *Emys orbicularis* and the otoliths of other species studied are of the same crystal form throughout the vertebrate class to which they belong.

LITERATURE CITED

Carlstrom, D. and H. Engstrom, 1955, Ultrastructure of Statoconia. Acta Oto-Laryng. 45: 14–18.

Romanoff, A. L. and A. J. Romanoff, 1949, The Avian Egg. John Wiley & Sons, New York, N. Y.

Tenaglia, G., 1926, Observations anatomiques macroscopiques et microscopiques sur les otolithes etudiés à frais. Arch. Ital. Biol. 77: 1–16.

Young, J. D., Structure and properties of the Testudinian egg shell. Proc. Zool. Soc. Lond. 120: 435–469.

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